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THE IMPORTANCE OF TRANSFERRIN-BOUND IRON  
FOR THE PROLIFERATION OF MOUSE  
T-LYMPHOCYTES IN VITRO

by

TRYFONIA MAINOU-FOWLER, B.Sc.

being a thesis submitted for the degree  
of Doctor of Philosophy in the Faculty of Science

University of Glasgow

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PUBLICATIONS

Work described in this thesis is included in the following publications:

1. Brock, J.H. & Mainou-Fowler, T. (1983) The role of iron and transferrin in lymphocyte transformation. Immunol. Today, 4, 347.
2. Mainou-Fowler, T. & Brock, J.H. (1985) Effect of iron deficiency on the response of mouse lymphocytes to concanavalin A: the importance of transferrin-bound iron. Immunology, In press.

# ABBREVIATIONS

Abbreviations used in the text include:

|                 |   |
|-----------------|---|
| $\alpha$ -      | anti-   |
| ANAE            | alpha naphthyl acetate esterase   |
| ANOVA           | analysis of variance  |
| Ab $\alpha$ TfR | antibody to transferrin receptor  |
| apoTf           | apotransferrin  |
| ATP             | adenosine triphosphate  |
| CMI             | cell-mediated immune  |
| ConA            | concanavalin A  |
| CoTf            | transferrin containing cobalt   |
| c.p.m.          | counts per minute   |
| DD              | group of mice fed on low-iron semisynthetic diet  |
| d-DW            | de-ionised distilled water  |
| DFO             | desferrioxamine   |
| DNA             | deoxyribonucleic acid   |
| DS              | serum from iron-deficient mice  |
| DTH             | delayed type hypersensitivity   |
| DW              | distilled water   |
| Fab             | fragment obtained by papain hydrolysis of immunoglobulin molecule which consists of one light and one heavy chain |
| FcR             | receptor for the Fc fragment of immunoglobulin molecule   |
| FCS             | foetal calf serum   |
| Fe(NTA)         | ferric nitrilotriacetate  |
| Fig.            | figure  |
| EPR             | electron paramagnetic resonance   |

|        |   |
|--------|---|
| h      | hour  |
| Hb     | haemoglobin   |
| HSA    | human serum albumin                                     |
| i.e.   | that is   |
| IgG    | the major immunoglobulin in the serum of Man.           |
| LPS    | lipopolysaccharide                                      |
| 2-me   | 2-mercaptoethanol                                       |
| min    | minute  |
| MnTf   | transferrin containing manganese                        |
| nd     | not done  |
| ND     | group of mice fed on laboratory diet                    |
| NMR    | nuclear magnetic resonance                              |
| ns     | not significant   |
| NTA    | nitritotriacetate                                       |
| p      | probability   |
| PBS    | phosphate buffered saline                               |
| PF     | group of mice paired to DD group                        |
| PHA    | phytohaemagglutinin                                     |
| PPD    | purified protein derivative                             |
| PWM    | pokeweed mitogen  |
| RNA    | ribonucleic acid  |
| r.p.m. | revolutions per minute                                  |
| SD     | group of mice fed on iron-containing semisynthetic diet |
| S.D.   | standard deviation                                      |
| sec    | second  |
| SEM    | standard error of the mean                              |
| SS     | serum from iron-sufficient mice                         |

|      |                                 |
|------|---------------------------------|
| t    | student's test statistic        |
| TCA  | trichloroacetic acid            |
| TIBC | total iron binding capacity     |
| Tf   | transferrin                     |
| Tris | Tris-(hydroxymethyl)methylamine |
| u.v. | ultraviolet                     |
| vit. | vitamin                         |
| vs   | versus                          |

#### Volume

|         |            |
|---------|------------|
| l       | litre      |
| ml      | millilitre |
| $\mu$ l | microlitre |

#### Weight

|         |           |
|---------|-----------|
| Kg      | Kilogram  |
| g       | gram      |
| mg      | milligram |
| $\mu$ g | microgram |
| ng      | nanogram  |

#### Concentration

|         |            |
|---------|------------|
| M       | molar      |
| $\mu$ M | micromolar |

#### Miscellaneous

|       |   |
|-------|---|
| A%MTf | transferrin A% saturated with metal M   |
| F     | variance ratio. Equals to: mean square error attributable to a factor been tested, divided by error mean square |
| mA    | milliampere   |

|                |   |
|----------------|---|
| $\mu\text{Ci}$ | microcurie  |
| $\text{nCi}$   | nanocurie   |
| $\text{pH}$    | reciprocal $\log_{10}$ hydrogen ion concentration |
| $\sigma^2$     | see definition on page 241                        |
| $\Sigma$       | Sum of  |
| $\text{w/v}$   | weight by volume                                  |
| $\text{v/v}$   | volume by volume                                  |
| $\bar{x}$      | mean of $x$ values                                |

Symbols

|        |                  |
|--------|------------------|
| /      | per              |
| >      | greater          |
| <      | less             |
| $\leq$ | less or equal to |



CHAPTER 1  
LITERATURE REVIEW

## 1.1. THE PROCESS OF LYMPHOCYTE TRANSFORMATION

### 1.1.1. General considerations

Vertebrates possess a surveillance mechanism, the immune system which specifically recognises and selectively eliminates foreign invaders (i.e. antigens). This immune protection is achieved by the cooperation of two systems the humoral and the cellular immune response, each one resulting from the activity of two populations of morphologically indistinguishable lymphoid cells; the B-cells which initiate the humoral, and the T-cells which initiate the cellular immune responses.

There are two fundamentally different forms of cellular immune responses depending on the type of T-cells involved. One type recognises and reacts with antigens on target cells. The second type is active in delayed type hypersensitivity reactions (DTH): binding of the antigen on the surface of T-cells triggers a state of activation characterized by cell-enlargement and also increased cellular activity, and leads to cell differentiation and division. However, literature data indicate that lymphocyte activation does not necessarily lead to proliferation and synthesis of deoxyribonucleic acid (DNA), i.e. activated lymphocytes have to be committed to DNA synthesis. Lymphocytes apart from being activated by antigens, can also become activated upon interaction with mitogens including plant lectins such as phytohaemagglutinin (PHA) and concanavalin A (ConA) (Chess et al., 1975; Ling & Kay, 1975), or they can also be activated by products of microorganisms (Taranta et al., 1969; Greaves & Janossy, 1972; Rodey et al., 1972). Mitogens

are polyclonal and, therefore, they induce a much greater response in lymphocytes in vitro than antigens, which activate only few clones of cells. Lymphocyte proliferation and differentiation is associated with secretion of substances (i.e. lymphokines) which attract and activate macrophages which, in turn, eliminate the antigen. T-lymphocyte activation and transformation, therefore, play the key role in inducing DTH reactions, and factors which affect either process will, in consequence, affect cell-mediated immune responses. Many factors have been shown to affect T-cell activation and proliferation among which is the existence of several kinds of accessory cells. These cells are necessary to present the antigen to the lymphocytes and also to secrete substances which enhance T-cell activation. Antigen- or mitogen-triggered lymphocyte proliferation also depends on factors which affect the degree of transformation-associated cellular activity. The elevated activity in proliferating cells reflects, primarily, increased synthesis of ribonucleic acid (RNA) and DNA and also synthesis of metabolically important compounds, proteins and enzymes, which include metalloenzymes and cytochromes. An important component for the synthesis and activity of these metalloenzymes and cytochromes is iron. Availability of iron during the synthesis of these molecules will, therefore, affect the rate of their production, and inadequate iron-delivery may be reflected in impaired proliferation of activated T-lymphocytes. It is this aspect of cellular activity, and its role in lymphocyte transformation which forms the basis of the work reported in this thesis.

### 1.1.2. Mitogenic activation of T-lymphocytes

Binding of the mitogen on the lymphocyte cell membrane is a crucial first step in the activation of lymphocytes as demonstrated by the finding that sugars which prevent binding of the lectin also inhibit lymphocyte activation and transformation (Borberg et al., 1968; Powell & Leon, 1970). This has led to the hypothesis that receptors containing an oligosaccharide moiety specific for the lectin exists on the lymphocyte membrane (Ling & Kay, 1975). Glycoproteins associated with lectin-binding have been isolated from the cell membrane of lymphocytes (Ling & Kay, 1975; Stobo, 1977), and they have been shown to contain the sequence:  $\longrightarrow$ galactose  $\longrightarrow$ sialic acid (Novogrodsky, 1975). Since lymphocytes bind similar numbers of lectin molecules irrespective of the type of lectin (Stobo et al., 1972; Bold et al., 1975), and glycoproteins isolated from the plasma membrane of lymphocytes by affinity chromatography with different lectins are essentially the same (Henkart & Fisher, 1975), it is concluded that a common receptor molecule for the mitogenic lectins may exist. Similarly, it has also been reported (Hellström et al., 1976) that T-lymphocytes have a common receptor for ConA and PHA, and recently Palacios & Möller (1981) have shown that OKT3 antibody, ConA and PHA trigger T-lymphocytes through the same receptors.

Binding of the mitogen does not necessarily lead to lymphocyte activation. A crucial number of mitogen molecules must bind to the lymphocyte surface in such a way as to

provide a sufficient surface stimulus, and also there must be a linkage between events occurring at the surface and inner mechanisms of cell-activation (Stobo et al., 1972 ). Literature data indicate that the proliferation of lymphocytes in response to mitogens (or antigens) is initiated by the cross linking of the receptors exposed on the cell-surface (Greaves & Janossy, 1972; Lindahl-Kiessling, 1972; de Petris, 1974; Rao, 1982). Interaction of ligands with surface receptors on lymphocytes leads to rapid changes in the surface membrane and to "cap" formation, as indicated by treating lymphocytes with fluorescein-labelled ConA at 37°C (de Petris, 1974; Rao, 1982). Since "cap" formation is found to be affected by cytoskeletal modulating agents such as colchicine (de Petris, 1974; Albertini et al., 1977) or cytochalasin B (de Petris, 1974; Yahara & Edelman, 1972), it has been suggested that "cap" formation is the result of the interaction of receptors with microtubules and microfilaments in the cell cytoplasm, and the disruption of these elements leads to alterations in the mobility of the surface receptors (Yahara & Edelman, 1972). Rao (1982) has shown that adenosine triphosphate (ATP)-dependent sliding of membrane-associated actin and myosin filaments was responsible for the accumulation of ConA-receptor complexes into a "cap" on the cell membrane, and they also showed that "cap" formation was associated with the degree of the mitogenic response. These results indicate that "cap" formation might trigger events which lead to proliferation. Polymerisation of actin in lymphocytes has also been implicated

as a possible signal for mitogenesis (Rao, 1984). Furthermore, Edelman (1976) has suggested that a signal for initiation of DNA synthesis is mediated by an assembly of interacting macromolecules consisting of cell surface receptors, microtubules and actin-containing microfilaments. However, the exact role of microfilament-formation and actin polymerization in the mitogenic (or antigenic) stimulation of lymphocytes remains to be explained.

In an attempt to further elucidate the cellular mechanisms involved in mitogen activation, studies of biochemical events associated with mitogen-activation of lymphocytes have been performed. These studies demonstrated that within minutes of addition of the mitogen, there is a marked increase in the membrane transport of small molecules (van den Berg & Betel, 1971; Ling & Kay, 1975) and ions (Crumpton et al., 1975; Ling & Kay, 1975) and also an increase in membrane-lipid turnover (Fisher & Mueller, 1968; Crumpton et al., 1975) and in the levels of cyclic adenosine monophosphate and enzymes (Smith et al., 1970; Ling & Kay, 1975). Histone acetylation also occurs (Hirschhorn et al., 1969). The rate of protein synthesis is increased, too, as indicated by quantitative cytochemical studies in small and activated lymphocytes and also by the increase in the rate of incorporation of labelled precursors into newly synthesised protein (Ling & Kay, 1975). The formation of large protein aggregates in stimulated lymphocytes appeared to be generated from pre-formed and newly synthesized proteins (Levy & Rosenberg, 1973). These early morphological and biochemical

events are thought to be involved in the activation of previously repressed genetic loci (Stobo, 1977).

### 1.1.3. Cellular requirement for activation of T-lymphocytes

Studies on more purified lymphocyte populations have suggested that the original idea that a lymphocyte could combine with its stimulant and then undergo activation is an oversimplification. It is now well established that lymphocyte activation by antigens or mitogens requires interaction with different cells. Both the direct interaction between lymphocytes brought into contact by agglutinating stimulants and interactions with adherent cells are involved.

#### 1.1.3.1. Lymphocyte-lymphocyte interaction

The formation of aggregates which occurs after the addition of almost any mitogen to lymphocyte cultures has been considered to be important in lymphocyte activation. Thus, agitation of stimulated cell cultures which prevented the formation of aggregates also inhibited the mitogenic response especially when the cultures were agitated early after addition of the mitogen (Peters, 1972). The inhibition of lymphocyte activation by hyaluronic acid (Darzynkiewicz & Balaz , 1971), and also the decreased rate of incorporation of labelled uridine into RNA in response to PHA when the cells were cultured in medium containing 0.2% agar (Peters, 1972) indicate that the reduced response of lymphocytes was probably the result of inhibition of an interaction between the cells in the culture. Cooperation among T-cell subsets has been found in synergistic interaction between murine thymus and lymph node cells (Piguet et al., 1975). The

response of FcR negative T-cells which were unresponsive to ConA was also increased by the presence of FcR positive cells (Stout & Herzenberg, 1975) and Ia<sup>-</sup> T-cells became responsive to ConA in the presence of Ia<sup>+</sup> cells (Frelinger, 1977). B-lymphocytes may also permit a helper effect on the response of T-cells to mitogens (Delespesse et al., 1976; Kasahara et al., 1979; Kin et al., 1979) or to protein A (Kasahara et al., 1980) and they are shown to permit mixed lymphocyte reactions between immunocompetent thymus cells (Dyminski & Smith, 1977). The consequence of this communication and what information is transferred from lymphocyte to lymphocyte remains to be fully elucidated.

#### 1.1.3.2. Lymphocyte-macrophage interaction

With the introduction of column filtration techniques for routine preparations of lymphocyte cultures with low levels of contaminating phagocytic cells, it was found that the response to many of the weaker lymphocyte stimulants such as antileucocyte antisera or staphylococcal filtrate was lost or greatly reduced when purified human lymphocytes were cultured in vitro (Oppenheim et al., 1968). The activity was restored by the addition of phagocyte-rich populations. Highly purified cultures of lymphocytes also showed a marked dependence on the presence of macrophages for their response to optimum concentration of PHA, and the number of adherent cells required to induce the response was very small, often no more than 0.5% of the total number (Levis & Robbins, 1970). Similarly, the proliferation of highly purified T-lymphocytes



in response to ConA and PHA (Rosenstreich et al., 1976) or to  $\text{NaIO}_4$  and galactose oxidase (Novogrodsky, 1975) was markedly enhanced upon incubation with macrophages. The blastogenic response of antigen-activated T-lymphocytes was also found to be dependent on the interaction of lymphocytes with macrophages (Waldron et al., 1973; Unanue, 1981). Further evidence for the macrophage-requirement in T-cell proliferation also comes from cytological observations which showed that sensitized lymphocytes adhered to macrophages in the presence of the PPD (Salvin et al., 1971). The importance of macrophages in T-cell proliferation was found to be due to the requirement by the lymphocytes of a soluble factor produced by the adherent cells, which functions as an essential activating signal (Gery et al., 1972; Hoessli & Waksman, 1975; Rosenstreich et al., 1976; Mizel & Ben-Zvi, 1980; Smith et al., 1980b; Palacios, 1982b; Maizel & Lachman, 1984). This lymphocyte-activating factor or interleukin-1 (IL-1) has been characterised and its function on lymphocyte proliferation (B- or T-cells) is now well established (also see 1.1.4). In mitogenic stimulation of T-cells, macrophages are essential mainly for the production of IL-1. Triggering is initiated by the interaction of the mitogen with its receptors on the T-lymphocytes (Palacios, 1982a). T-cell activation by antigens, however, requires macrophages not only for IL-1 production but also for the presentation of the antigen to the T-cells as indicated experimentally using antibody reactive against HLA-DR (or Ia in mice) antigens (Frolinger et al., 1976;

Albrechtsen et al., 1977; Palacios, 1982b).

1.1.4. The "commitment" of lymphocytes to proliferation

It is now well established that maximum commitment of lymphocytes to DNA synthesis requires some 18 h exposure to the mitogen (Wedner & Parker, 1976). Shorter exposure times do, however, result in blastogenesis but cells return to their resting stage within a few minutes after the removal of the lectin (Sell & Sheppard, 1974). Furthermore, supra-optimal doses of ConA which inhibit DNA synthesis, nonetheless "prime" T-cells to entry into S phase i.e. stimulate them to enter to G<sub>1</sub> phase of the cell cycle (McClain & Edelman, 1976). These results indicate that activation of lymphocytes is not synonymous with commitment to DNA synthesis, and factors other than the binding of the lectin and the presence of the co-stimulator IL-1, are required before cells undergo DNA synthesis.

It has been found that T-cells activated with a mitogen or antibody produce interleukin-2 (IL-2), a growth factor which is responsible for the maintenance of their continuous proliferation (Morgan et al., 1976; Bonnard et al., 1979; Larsson & Coutinho, 1979; Smith, 1980). Interleukin-2 supported the growth of activated T-cells (Larsson & Coutinho, 1979; Smith, 1980; Palacios et al., 1981) although resting T-cells became sensitive to IL-2 when they were activated (Palacios et al., 1981). Furthermore, the production of IL-2 was associated with cell proliferation since OKT8 antibody inhibited IL-2 production and proliferation of OKT8<sup>+</sup> human T-cells (Welte et al., 1983). It has also been

reported that in the mouse, the production of IL-2 is associated with  $Ly1^{+}2^{-}3^{-}$  T-cells, while  $Ly1^{-}2^{+}3^{+}$  cells respond to it by proliferation (Smith, 1980). The production of IL-2 by stimulated T-cells has been associated with the expression on the cell membrane of IL-2 receptors and this has been confirmed biologically as activated T-cells effectively absorb IL-2 from conditioned media preparation (Bonnard et al., 1979; Robb et al., 1981). In addition, using a recently described monoclonal antibody, anti-Tac, that specifically recognizes the IL-2 receptor (Leonard et al., 1982; Depper et al., 1983) it has been shown that acquisition of the receptor depends on lectin stimulation (Depper et al., 1983). The literature data therefore indicate that production of IL-2 receptors is associated with activated lymphocytes and that their acquisition by T-cells may be one of the functions of lectin interaction with the cells. However, given a central role of macrophages and IL-1 in immunoregulation, a question of importance concerns what role these cells play in the expression of IL-2 receptors. There are no data so far suggesting that concomitant IL-1 exposure may change the kinetics of acquisition or the density of IL-2 receptors. It is known, though, that IL-1 promotes the production of IL-2 by activated T-cells (Smith et al., 1980a; Hunig et al., 1983) and that both these factors play a crucial role in T-cell proliferation. Thus two different models have been postulated to describe the T-cell cycle.

1) Wagner et al. (1980) postulated that  $Ly1^+2^-3^-$  cells produce IL-2 in response to the combined action of antigen or mitogen and IL-1, and then IL-2 induces DNA synthesis in precursors of  $Ly1^-2^+3^+$  cells which have been activated by the antigen to express receptors for IL-2.

2) An alternative model is provided by Lafferty et al. (1980) according to which there are two stages:

1. Resting T-cells (either  $Ly1^+2^-3^-$  or  $Ly1^-2^+3^+$ ) are stimulated by the combined action of antigen or mitogen and IL-1 derived from accessory cells.

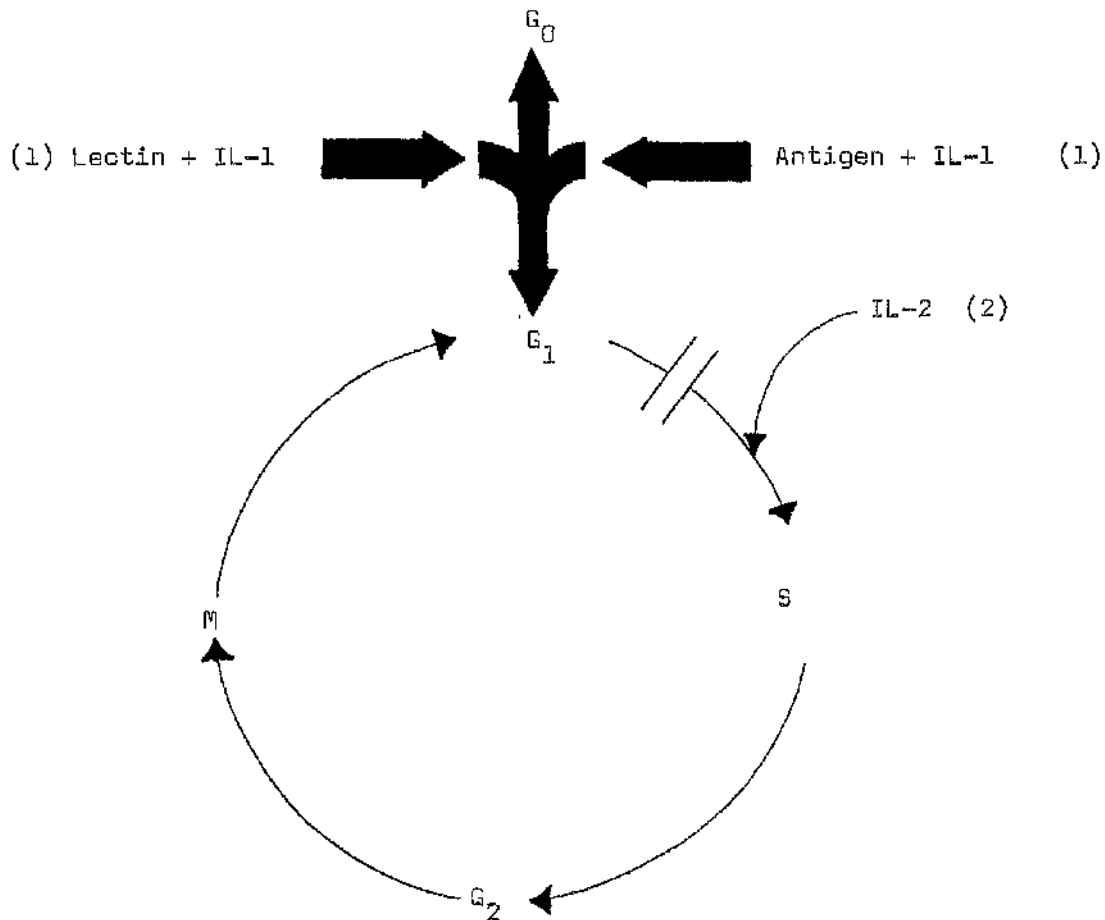
2. The above sensitize the cell to progress to a stage where the specific requirement for antigen or the mitogen remains but a signal is provided by IL-2 which causes the activated cell to be committed to DNA synthesis.

In conclusion, the lymphocyte stimulation by antigens or mitogens can be divided into two stages, the activation stage ( $G_0$  to  $G_1$ ; Fig. 1) and the commitment stage ( $G_1$  to S). Immunological specificity is preserved in the initial activation event, while clonal growth and differentiation can then be maintained by IL-2, which acts in  $G_1$  phase from which cells are committed to enter the phase of DNA synthesis (S).

#### 1.1.5. DNA synthesis by activated lymphocytes

The nature of the signal for the initiation of DNA synthesis is not yet fully understood, but it is thought to be a cytoplasmic function. The DNA synthesis observed after activation of lymphocytes is due simply to the normal replication

Figure 1: Cell-cycle control in T-cells.



Resting T-cell ( $G_0$ ) is activated into  $G_1$  by signal (1), and it will arrest somewhere in  $G_1$  unless further stimulation by IL-2 (signal (2)) induces commitment to DNA synthesis.

(From Klaus & Hawrylowicz, 1984).

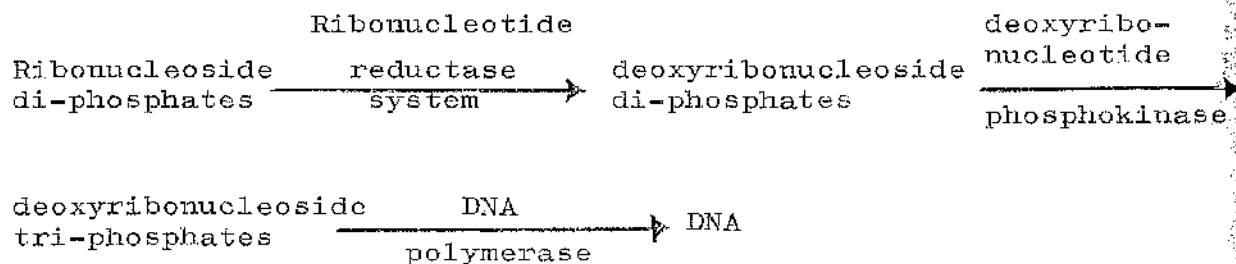
of the chromosomal DNA required for mitosis, and follows the normal semi-conservative replication. DNA synthesis is accompanied by synthesis of ribosomes since low concentrations of actinomycin which inhibit synthesis of rRNA also inhibited DNA synthesis (Kay et al., 1969). The induction of several enzymes is also associated with the initiation of DNA synthesis. Thus increased DNA-polymerase activity was observed after PHA stimulation and it was followed by increased incorporation of  $^3\text{H}$ -thymidine by the stimulated cells (Loeb & Agarwal, 1971). Increased activity of other enzymes involved in DNA synthesis has also been reported (Ling & Kay, 1975).

The initiation of DNA synthesis in lymphocytes also seems to have some requirement for certain metals which are involved in enzyme activity. These metals include zinc (Chesters, 1972) and iron (Wrigglesworth & Baum, 1980), and the requirement for iron is dealt with in detail in the following section.

#### 1.1.6. The role of iron in DNA synthesis

Although iron is required for many enzymes and coenzymes, the most important requirement as far as lymphocyte transformation is concerned appears to be linked with an iron requirement for DNA synthesis. Iron is essential component of the enzyme ribonucleotide reductase (Brown et al., 1969; Moore et al., 1970). This enzyme is a component of a cytoplasmic enzyme system which catalyses the reaction of the four ribonucleotides to their corresponding deoxy-

ribonucleotides which are essential for the synthesis of DNA. Thus:



In mammalian tissues the reductase system consists of three major protein components; a ribonucleotide reductase, a thioredoxin polypeptide and a thioredoxin reductase. The reduced form of thioredoxin provides the source of electrons for the ribonucleotide reaction. Ribonucleotide reductase comprises two non-identical protein subunits. One subunit contains two iron atoms probably both in high spin ferric form. An electron spin resonance signal can be detected in the subunit but the characteristics are those of an organic free radical. This may be the site for thioredoxin interaction, which is not yet known. Iron seems not to participate as an electron carrier in the reaction but it is necessary for the activity of the enzyme, and Atkin et al. (1973) have suggested that the function of the iron is to help to generate and stabilize the organic radical intermediate during catalysis. The other subunit binds to the substrate (i.e. the ribonucleotides).

The importance of ribonucleotide reductase in DNA synthesis was shown by Brockman et al. (1971) who reported impaired incorporation of <sup>3</sup>H-thymidine into DNA by a leukaemia cell-line when the cells were cultured in medium

containing inhibitors of ribonucleotide reductase enzyme. In addition, it has been shown that iron taken up by HeLa cells was transferred to the nucleus and embedded into the chromosomes during cell division (Robbins & Pederson, 1970) and also desferrioxamine (DFO) which is an iron chelator, inhibited DNA synthesis. Hoffbrand et al. (1976) have described the mechanism by which DFO inhibits DNA synthesis, and this is discussed in detail in Section 1.3.3.

In conclusion, availability of iron affects the activity of the iron-containing ribonucleotide reductase enzyme which is essential for the synthesis of DNA. Since iron is normally delivered to cells by the iron-transport protein transferrin, it seems likely that the increased iron requirement to rapidly dividing cells will lead to an increased need for transferrin-bound iron, hence the importance in cell proliferation. This aspect has been the subject of the work reported in this thesis, and the properties of transferrin are discussed in the following section.



## 1.2. TRANSFERRIN

### 1.2.1. General considerations

Iron is essential to all forms of life. The flow of electrons through the biosynthetic pathways, the activation of molecular oxygen, hydrogen and nitrogen, the binding of oxygen to haemoglobin, myoglobin and haemerythrins and the decomposition of noxious derivatives of oxygen, are all reactions requiring iron (Wrigglesworth & Baum, 1980). However, although iron is important in life, it can also present hazards to organisms. In aqueous solution iron has access to two different oxidation states; ferrous ( $\text{Fe}^{2+}$ ) and ferric ( $\text{Fe}^{3+}$ ). Under physiological conditions the more stable form is the ferric, while ferrous iron is readily oxidised by molecular oxygen to ferric iron. Since ferric iron is insoluble, organisms have evolved specific iron-sequestering molecules to maintain the element in a soluble form which is readily available for transport and biosynthetic reactions. In vertebrates, these iron-binding functions are met by transferrin, whose main function is iron transport, and ferritin which is essentially an iron-storage protein (Jandl & Katz, 1963; Aisen & Listowsky, 1980).

Transferrin is a glycosylated  $\beta$ -globulin which has the ability to bind two iron atoms reversibly. In Man, serum contains 2-4 mg/ml transferrin of a saturation with iron of about 30%. The transferrin concentration and iron saturation vary, however, with pathological conditions (Morgan, 1981). In vertebrates, transferrin is synthesized mainly in hepatocytes, although lymph nodes, peripheral

blood lymphocytes and other tissues have also been implicated (Morgan, 1981).

#### 1.2.2. Structure of transferrin

Recent elucidation of the amino acid sequence of human transferrin showed that the molecular weight including the carbohydrate chain is 79,550 (MacGillivray et al., 1982). Physical studies have given similar values for the plasma transferrins of other vertebrates (Morgan, 1981). The shape of the molecule has the form of prolate ellipsoid with axial ratio of 1:2 for iron-saturated transferrin (Rosseneu-Montreff et al., 1971) and 1:2.5 (Rosseneu-Montreff et al., 1971) or 1:3 (Bezkorovainy & Rafelson, 1964) when the protein is iron-free.

Human transferrin consists of a single polypeptide chain together with two glycan moieties attached to the C-terminal region of the peptide chain. The striking feature of the overall organisation of the molecule, as revealed by X-ray studies of transferrin crystals (Gorinsky et al., 1979), is that the polypeptide chain is folded into two globular sub-structures or domains each one containing a cleft which corresponds to the iron binding site of each molecule (Morgan, 1981). Within each domain there are disulphide bridges (Elleman & Williams, 1970; Williams, 1982), and a bridging region limited to a maximum of seven residues join the two domains together (MacGillivray et al., 1982). These domains, as indicated by studies of iron-binding fragments of bovine transferrin (Brock & Arzabe, 1976) or ovotransferrin (Williams, 1974; 1975) differ from each other

physically and antigenically. Although there is a difference in the physical properties of the two domains (Aisen & Listowsky, 1980), the functional significance of the presence of the two domains with separate iron-binding sites is uncertain. There might, however, be an evolutionary advantage of the two-domain structure, which would reduce loss of protein through glomerular filtration (MacGillivray et al., 1982). The polypeptide chain of human serum transferrin consists of 678 amino acids (MacGillivray et al., 1982). There is an internal 40% homology between residues 1-336 and 337-678, each range representing an iron binding site, suggesting gene duplication of an ancestral protein with a single domain (MacGillivray et al., 1982). Amino acid sequence is also known for chick transferrin (Jeltsch & Chambon, 1982; Williams et al., 1982a) which also shows internal homology (Jeltsch & Chambon, 1982).

Human transferrin contains approximately 6% carbohydrate disposed on two identical and nearly symmetrical branched heterosaccharide chains each terminating in one or more sialic acid residues attached to a galactose residue (Dorland et al., 1977; Morgan, 1981). The carbohydrate moieties are attached to the polypeptide chain by a  $\beta$ -1 glycosidic linkage via an asparagine residue (Aisen, 1980). This most frequent structure is a biantennary glycan structure although a triantennary structure has also been reported (Krusius & Finne, 1981). Transferrins isolated from other species also contain a single (Heaphy & Williams, 1982a) or two glycan chains (Hudson et al., 1973; Iwase &

Hotta, 1977)). Despite the considerable knowledge of the structure of the carbohydrate moieties of transferrin, little is known about their function. The glycan chains may be important in determining the rate of elimination of the protein from the circulation since removal of sialic acid in many glycoproteins leads to rapid uptake and catabolism in the liver. However, there is no conclusive evidence for the involvement of the glycan in the catabolism of transferrin (Aisen, 1980).

### 1.2.3. Metal binding

#### 1.2.3.1. Iron binding

Iron free molecule of transferrin (i.e. apotransferrin) reacts with ferric ions to produce a salmon pink complex which has maximum absorbance at 467-470 nm. Each iron atom binds to and occupies each site of the polypeptide chain (i.e. domain) so strongly that it is effectively "locked in". The domains become more compact when iron is bound (Bezkorovainy & Rafelson, 1964) and the molecule becomes less susceptible to proteolytic degradation and denaturation (Brock et al., 1976; 1980). Iron binds as the ferric form although there have been reports that ferrous iron can also bind but with lower affinity, and in the presence of oxygen it is converted to the ferric iron-complex (Kojima & Bates, 1981).

The affinity of iron binding by transferrin is pH-dependent and it is maximal above pH 7.0 (Morgan, 1981).

When the pH is reduced, the affinity becomes lower, the iron starts to dissociate from the molecule at about pH 6.5, and it is completely released at pH 4.5 (Morgan, 1981). In human transferrin, the iron is dissociated from the molecule in a biphasic manner depending on the pH, and half of the iron is released at about pH 5.4, whereas the remainder is lost when the pH drops below 5.0 (Princiotta & Zapolski, 1975). This biphasic release of iron suggests that there might be a difference in the iron-binding properties of each site. Cannon and Chasteen (1975) and Zweier (1978) showed that ferric ions have a tendency to bind preferentially at one site of the transferrin molecule at a pH above 7.2 thus referred to as the acid labile site, the other acid stable site, becomes occupied at pH 6.0-7.1. These sites are shown to correspond to N- and C-terminal sites of the molecule (Evans & Williams, 1978). The removal of iron from one site over the other also depends on salt concentration, and it has been reported that chloride facilitates removal of iron from the C-terminal site but retarded its removal from the N-terminal site (Van Eijk et al., 1978). Addition of chelating agents in vitro as a source of iron also revealed preferential binding to one site depending on the pH of the medium (Brock & Arzabe, 1976; Williams et al., 1978). The use of EPR spectroscopy revealed quantitative differences between the C- and N-terminal site of ovotransferrin (Keung et al., 1982). These investigators reported that the  $\alpha$ -helical content of the C-site was twice that of the N-site, and that

major differences in the content of histidine, alanine and methionine also existed.

The binding of each iron to transferrin requires the synergistic binding of an anion (Aisen et al., 1967; Price & Gibson, 1972; Bates & Schlabach, 1975; Gelb & Harris, 1980) which is usually either a carbonate or bicarbonate; the exact nature of the anion is still in dispute (Gelb & Harris, 1980; Zweier et al., 1981). In the absence of a synergistic anion, the iron does not bind specifically, and non-specific binding in the form of ferric-hydroxide polymers has been reported (Bates & Schlabach, 1975). Release of the anion destabilizes the complex of iron-transferrin allowing release of iron. A large number of anions may be substituted for (bi)carbonate in the specific binding of iron to transferrin (Young & Perkins, 1968; Aisen et al., 1973; Egyed, 1973; Schlabach & Bates, 1975). These anions which include oxalate, malonate, pyruvate, glycolate, lactate, malate and gluconate, form complexes with transferrin that are less stable than those containing (bi)carbonate. Chelating agents such as nitrilotriacetate (NTA) can also substitute for (bi)carbonate. It has been reported that binding of Fe(NTA) to apotransferrin results in the formation of a ternary iron- and chelate-containing complex of transferrin, in which the iron is more labile than when (bi)carbonate is used. Addition of (bi)carbonate does not displace the chelate immediately but induces the formation of a quaternary complex of transferrin with iron, chelate and (bi)carbonate from which the chelate is later released (Bates, 1982).

Iron-binding by transferrin does not involve any specific prosthetic groups. The metal-binding sites are the consequence of folding of the polypeptide chain to produce two spatial domains which are stabilized by disulphide bonds and other secondary interactions (Feeney & Komatsu, 1966). The iron, when bound to transferrin possesses an octahedral configuration with six ligands, one of which is bound to the synergistic anion and another to a molecule of water (Zweier & Aisen, 1977). The remaining ligands bind to the protein. Controversial data exist concerning the identity and the number of protein ligands. Thus, it has been suggested that either three (Warner & Weber, 1953; Windle et al., 1963; Gelb & Harris, 1980) or two (Tan & Woodworth, 1969) tyrosine residues are involved in each iron-binding site. There is also evidence that either one (Zweier & Aisen, 1977) or two (Rogers et al., 1977) histidine residues are involved as iron-binding ligands. Arginine and lysine have also been implicated, but their involvement reflects binding to the synergistic anion rather than binding to iron (Zweier & Aisen, 1977).

#### 1.2.3.2. Binding of metals other than iron

Transferrin can bind metals other than iron, although iron is probably the only metal bound to transferrin in vivo. Specific binding of these metals should fulfil the following criteria (Aisen, 1980):

1. Only two metal ions should bind per molecule of transferrin.
2. One (bi)carbonate (or other anion) should bind for each metal.

3. Binding of the metal to iron-saturated transferrin should not occur.

Metals which bind to transferrin and fulfil most of the above criteria include:  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{V}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Co}^{3+}$ ,  $\text{Ga}^{3+}$ ,  $\text{Mn}^{3+}$  (Feeney & Komatsu, 1966; Aisen et al., 1969; Tan & Woodworth, 1969; Woodworth et al., 1970; Harris et al., 1974; Zweier, 1978). Lanthanide ions such as  $\text{Tb}^{3+}$ ,  $\text{Eu}^{3+}$ ,  $\text{Nd}^{3+}$ ,  $\text{Ho}^{3+}$  (Mears & Ledbetter, 1977; O'Hara et al., 1981) and actinides  $\text{Th}^{4+}$ ,  $\text{Pu}^{4+}$  (Pecoraro et al., 1981) also bind with the same stoichiometry as iron and probably occupy the same sites. Metal displacement studies and relative binding affinities of metal complexes of transferrin showed that the order of binding was  $\text{Fe}^{3+} > \text{Cr}^{3+}$  and  $\text{Cu}^{2+} > \text{Mn}^{3+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+}$  (Tan & Woodworth, 1969). Feeney & Komatsu quoted unpublished work of Inman (1956) according to which  $\text{Co}^{3+}$  had a greater binding affinity for transferrin than  $\text{Fe}^{3+}$  and, therefore,  $\text{Co}^{3+}$  could displace  $\text{Fe}^{3+}$  in the transferrin molecule. However, recent work using NMR spectroscopy (Zweier et al., 1981) showed that  $\text{Fe}^{3+}$  will displace  $\text{Co}^{3+}$ , and therefore the earlier observation of Inman probably was incorrect.

Thus, although binding of metals other than iron to transferrin is probably non-physiological, it has served as a useful tool in spectroscopic studies of the transferrin molecule.



#### 1.2.3.3. Binding and release of iron from transferrin in vitro

In the presence of (bi)carbonate, iron binds to either site of transferrin. Kinetic rather than thermodynamic factors govern the distribution between the sites (Van Eijk et al., 1978). The distribution of iron between the sites is affected by the pH and salt concentration of the solution (Williams et al., 1978; Chasteen & Williams, 1981; Williams et al., 1982b). In vitro, loading of transferrin with iron may create problems. Although the association constant of iron and transferrin is high ( $10^{20} \text{ M}^{-1}$ ) so that iron binding can easily occur,  $\text{Fe}^{3+}$  ions can exist in solution at neutral pH only at very low concentrations and in excess of  $2.5 \times 10^{-18} \text{ M}$ , are insoluble. At higher concentrations, the iron is hydrolysed and polymers are formed which react slowly with transferrin (Bates & Schlabach, 1973), and furthermore they can react with hydroxyl groups of the transferrin molecule, thus causing non-specific binding (Bates & Schlabach, 1973). The problem of non-specific binding of iron, when ferric salts are used, can be avoided by using iron chelators such as nitrilotriacetate or citrate. When using citrate to ensure absence of polymers, the reactive species should be of low molecular weight, and this is achieved by using a 20-fold excess of citrate ions. Nitrilotriacetate, when used at neutral pH at a 1:4 ratio of iron to chelate, also results in rapid iron-binding (Schlabach & Bates, 1975). However NTA can also occupy the anion-binding site (see 1.2.3.1) and this can be avoided by adding (bi) carbonate which will slowly displace the NTA.

Iron removal from transferrin in vitro is usually achieved with low pH to destabilize the iron-protein bond and an iron chelator such as citrate to accept the released iron. Even at physiological pH, however, iron can be eliminated from the protein when a more powerful iron-binding molecule such as desferrioxamine is available to bind the released iron (Aisen, 1980). However, since the iron-transferrin bond is so strong that spontaneous dissociation of iron from the protein is difficult, mediating agents have to be used to facilitate the transfer of iron from protein to DFO. Effective mediating agents include NTA and ketomalonate (Aisen, 1980).

#### 1.2.4. Function of transferrin

##### 1.2.4.1. Introduction

The most important and certainly the most studied role of transferrin is the transport of iron among the sites of absorption, storage, utilisation and excretion, most iron being delivered to erythroid precursors for the synthesis of haem. Small but significant quantities of iron are also delivered to non-erythroid cells, especially the rapidly dividing cells. During pregnancy, transferrin also acts as an iron transport molecule to the placenta for transport to the foetus. The mechanism by which transferrin accepts and donates iron has been extensively investigated, but is still not fully understood. Most of the original investigations concerning the donation of iron to cells by transferrin were carried out using reticulocytes, but it is now known

that most of the features apply to non-erythroid cells such as lymphocytes.

Another important function of transferrin is associated with antimicrobial activity. By restricting the availability of iron for microbial metabolism, transferrin may have an important role in the defence against infection (Pearson & Robinson, 1976). Intracellularly, transferrin may also function in the killing of phagocytosed bacteria via a catalytic pathway involving the generation of hydroxyl radicals from superoxide and peroxide (Motohashi & Mori, 1983).

#### 1.2.4.2. Events in the transferrin-cell interaction

It is now generally considered that several steps are involved in the uptake of transferrin-bound iron by erythroid and non-erythroid cells. These are:

1. binding of transferrin to specific receptor sites on the cell membrane
2. entry of transferrin into the cell by endocytosis
3. removal of iron from transferrin
4. release of apotransferrin from the cell into the surrounding medium.

These are discussed in more detail below.

#### 1.2.4.3. Interaction of transferrin with membrane receptors

The existence of receptors on reticulocyte cell membranes was first noted by Jandl et al. (1959) who found that trypsinised red cells could no longer bind transferrin or take up iron from transferrin. They inferred that a receptor specific for transferrin existed on the cell membrane which

was degraded by proteolysis. Later Garrett et al. (1973) demonstrated transferrin-binding activity in detergent-solubilised reticulocyte stroma, probably due to receptors. Since then, many studies have been reported describing methods for the isolation and characterisation of the transferrin receptor, but results have been controversial. Thus, first estimates of the molecular weight have ranged from 18,000 - 450,000 (Aisen, 1983). Discrepancies in the exact values of the association constants for transferrin-receptor have been reported, too. Most of these controversial results arose due to using transferrin and cells from different species, different methods for isolating the receptors, or heterogeneity of cell suspensions. Studies, on non-erythroid cells have generated less controversial results. These reports indicate that the receptor is a glycoprotein (Omary & Trowbridge, 1981; Newman et al., 1982; Schneider et al., 1982) of molecular weight 170,000 - 200,000 and that it exists as a disulphide-bonded dimer, each subunit containing three glycan chains of molecular weight 95,000 (Seligman et al., 1979; Goding & Burns, 1981; Goding & Harris, 1981; Sutherland et al., 1981; Trowbridge & Omary, 1981; Schneider et al., 1982), and consisting of galactose, N-acetyl glucosamine and sialic acid (Newman et al., 1982). The transferrin receptor has been shown to contain palmitic acid covalently attached close to the cell membrane-associated part of the receptor molecule (Omary & Trowbridge, 1981; Newman et al., 1982) and it is

also phosphorylated, predominately on serine residues (Schneider et al., 1982). Part of the receptor can also be cleaved from the cell surface by proteolysis yielding a fragment of molecular weight 70,000 which retained the transferrin-binding sites, suggesting that the receptor is embedded in the cell membrane and the major part is exposed to the extracellular environment (Schneider et al., 1982). Transferrin receptors have been isolated from different cells including placenta cells, reticulocytes, kidney tissue and tumour cells, and so far no structural differences between them have been reported.

The expression of receptors on the cell membrane is associated with increased requirements for iron. The rate of iron uptake from transferrin during erythroid cell development was found to correlate closely with the number of transferrin receptors expressed on the cell membrane (Iacopetta et al., 1982), and this number decreased on cell maturation (Nunez et al., 1977; Frazier et al., 1982; Parmley et al., 1983). In non-erythroid cells, the transferrin receptor has also been associated with increased need for iron during cell proliferation. Thus the cell membrane antigen associated with proliferation and recognised by monoclonal antibody OKT9 is the transferrin receptor (Sutherland et al., 1981; Trowbridge & Omary, 1981). In addition, transformed lymphoid cell lines (Larrick & Cresswell, 1979b; Galbraith et al., 1980a; Makino et al., 1983) and lymphoblasts (Brock & Rankin, 1981) express a

higher number of transferrin receptors than normal resting lymphocytes. The percentage of cells to which transferrin binding was detected was found to correlate with the degree of lymphocyte DNA synthesis in response to mitogens (Galbraith et al., 1980b). Increased expression of transferrin receptors is also associated with malignancies. Thus, expression of a large number of receptors in leukaemic cells (Larrick & Logue, 1980) and in carcinomas, sarcomas and samples from cases of Hodgkin's disease (Gatter et al., 1983) has been reported. Additionally, Musgrove et al. (1984) observed large numbers of transferrin receptors in human tumour cells in culture, and showed that the expression of the receptors was related to the cell cycle phase thus reflecting the proliferative activity of the cells. The association of the expression of transferrin receptors with an increased requirement for iron by the proliferating cells is also justified by the observation that during differentiation when there is a decreased requirement for iron, the number of receptors on the cell membrane also decreases (Makino et al., 1983).

The binding of transferrin to the receptor is normally essential for the uptake of iron by the cells. Evidence that cellular iron uptake from transferrin is dependent on transferrin binding to the receptor comes from the observation that proteolytic digestion of reticulocytes leads to a reduction in the rate of iron uptake by the cells (Hemmaplardh & Morgan, 1976) and monoclonal antibodies to the transferrin receptor which inhibit binding of transferrin also inhibit

iron uptake and cell growth in vitro (Trowbridge et al., 1982; Trowbridge & Lopez, 1982). Monoclonal antibodies which do not affect transferrin binding do not affect uptake of iron (Sutherland et al., 1981; Trowbridge & Lopez, 1982). However, iron uptake not involving transferrin binding to the receptor, has also been reported (see 1.2.4.4).

The reaction between transferrin and detergent-solubilised receptor is dependent on pH, presence of  $\text{Ca}^{2+}$  and the iron content of transferrin (Morgan, 1981). The receptor is specific for transferrin, since binding of iron-saturated  $^{125}\text{I}$ -transferrin was competitively inhibited by iron-saturated unlabelled transferrin but not by other proteins (Larrick & Cresswell, 1979b). Studies with a number of different cell types show that diferric transferrin has a higher affinity for the receptor than apotransferrin at neutral pH (Jandl & Katz, 1963; Young & Aisen, 1981; Hasogawa & Ozawa, 1982; Kohgo et al., 1983; Ward et al., 1983). The functional advantage of the greater affinity of iron-containing transferrin than apotransferrin for the receptor is that apoTf will not greatly interfere with the iron uptake process by the cells. However, apo might sometimes interfere with the iron uptake process if the saturation of transferrin with iron were very low.

It is not yet known what part of the receptor is important in the binding of transferrin. However, recent studies showed that transferrin binding to the receptor was decreased when the receptor was treated with galactosidase or in the presence of carbohydrates such as galactose and

N-acetyl galactosamine (Steiner, 1980) indicating that the glycan moiety of the receptor is implicated. Even less is known about the moiety of transferrin which binds to the receptor. The carbohydrate part of the molecule is probably not involved since ovotransferrin and chicken serum transferrin which have different carbohydrate contents donate iron at the same rate to chick erythroid precursor cells (Keung & Azari, 1982). A factor which has been found to be important in the binding of transferrin and iron donation is the integrity of the transferrin molecule, since monoferric fragments of ovotransferrin and bovine transferrin had little ability to bind and donate iron to chick embryo cells or rabbit reticulocytes, respectively (Esparza & Brock, 1980 ; Brown-Mason & Woodworth, 1982).

It is now known that the transferrin receptor is required for the uptake of iron, although the exact way of the iron uptake process has not yet been fully elucidated. One possibility is that a kind of interaction of the transferrin-iron complex with the receptor may be required for iron release from the protein, although no experimental data are so far available to support this hypothesis. Another function, which has been extensively investigated, is that the receptor plays an essential role in the internalisation of transferrin-bound iron. This function as well as the actual process of endocytosis will be discussed in more detail below.



#### 1.2.4.4. Internalisation of transferrin

Receptor binding of compounds which exhibit capping or patching is a phenomenon which leads to internalisation of these compounds via coated pits. This phenomenon which is observed in many systems such as in the binding of mitogens on the surface of lymphocytes during cell activation (Stobo, 1977; Rao, 1982) or in the binding of antigens to the surface immunoglobulin on B-lymphocytes (Raff & de Petris, 1973), requires multi-subunit membrane receptors as well as binding of divalent ligands. Receptor-bound transferrin exhibiting patching or capping in mitogen stimulated lymphocytes (Galbraith & Galbraith, 1980) or in reticulocytes (Harding et al., 1983; Iacopetta et al., 1983) has been reported. Furthermore, Goding & Harris (1981) using 2-dimensional electrophoresis showed that the transferrin receptor is the major multi-subunit component in the cell membrane in murine T-lymphocytes. Transferrin also acts as a divalent ligand since monoferric fragments do not donate iron (Brock, 1981). These observations suggest that transferrin-bound iron is taken into the cell by receptor-mediated endocytosis, the iron is released from the transferrin and apotransferrin is then recycled to the exterior by the reverse process. Morphological data using colloidal gold bound to transferrin (Light & Morgan, 1982) or studies on the subcellular localisation of transferrin labelled with ferritin (Hemmaplardh & Morgan, 1977), peroxidase (Hemmaplardh & Morgan, 1977; Hopkins, 1983) or

Iodine-125 (Lamb et al., 1983; Nunez & Glass, 1983) showed that transferrin in various cell-types is internalised by endocytosis. Treatment of cells with pronase or metabolic inhibitors or heating at 46°C, processes which inhibit endocytosis, caused a reduction in the number of intracellular gold particles (Light & Morgan, 1982). Furthermore, Enns et al. (1983) studied endocytosis of transferrin in human erythroid cell lines using rhodamine-labelled transferrin and fluorescein-labelled Fab fragments of goat antireceptor IgG, and showed that transferrin and its receptor were internalised as aggregates and comigrated to the same structures within the cell. Hopkins and Trowbridge (1983) also showed that transferrin and the transferrin receptor were internalised and the complexes were transferred from coated pits on the cell surface to a system of cisternae in the peripheral cytoplasm and finally to small lysosome-like vesicles. Extraction of complexes using Triton-x and purification through a Sepharose-transferrin column, showed that the internalised transferrin remained bound to the transferrin receptor during the intracellular process. Additionally, Ciechanover et al. (1983) showed that exposure of the transferrin-receptor complex to medium of pH less than 5.0 resulted in dissociation of iron but that apo-transferrin remained bound to the receptor. When the pH was raised to 7.0, as would occur when an acidic intracellular vesicle fuses with the plasma membrane, apotransferrin rapidly dissociates from the receptor. Taken together these observations indicate that iron-containing transferrin

enters the cell by receptor-mediated endocytosis, the transferrin remains bound on the receptor throughout the endocytic cycle and the transferrin is exocytosed in a reverse process after the release of iron. Whether such internalisation is absolutely necessary before transferrin-bound iron is available to the cell remains unclear, and there have been studies indicating that iron may be taken up without being endocytosed. Thus, Woodworth et al. (1982) reported that the highly-specific microtubule inhibitor nocodazole did not affect the rate of iron uptake from transferrin by reticulocytes suggesting that endocytosis of transferrin is not essential for iron uptake. Similarly, Fielding & Speyer (1977) using a different approach found that low concentrations of sulphhydryl inhibitors which act by reducing cell membrane permeability depressed removal of iron from transferrin by reticulocytes by 50%. Since this inhibitor is thought to act on the cell surface without penetrating the cell, they considered that iron was dissociated from the transferrin at the cell surface and was then transferred into the cell, probably by a membrane component acting as an iron recipient. No such component was identified at the time. However, Glass et al. (1980) isolated transferrin- and iron-binding proteins from the membrane of reticulocytes and postulated that transferrin first binds to the cell membrane and then donates its iron to an iron acceptor membrane moiety that subsequently delivers iron to the cytosol. An iron-binding membrane protein in human melanoma cells which exhibits primary sequence-homology with transferrin has recently been

identified (Brown et al., 1982) and may correspond to the component described by Glass et al. (1980).

In conclusion, there are data which indicate that transferrin is taken up by receptor-mediated endocytosis. However, there is also evidence indicating that there may be a plasma membrane-mediated release and transfer of iron to the interior of the cells. The relative importance of these mechanisms still remains to be resolved, although it has been suggested that both processes may operate (Zaman et al., 1980), the extent to which each process operates depending on the type of cell involved.

#### 1.2.4.5. Release of iron from transferrin

Several mechanisms for iron-release from transferrin have been postulated, but the exact mechanism has not yet been fully understood. The elucidation of the mechanism involved in the removal of iron from transferrin has been complicated by the controversy about whether endocytosis is a requirement for the release of iron. Thus, it has been proposed that conformational changes in transferrin while bound to the cell membrane result in iron release to a membrane iron-binding component (see 1.2.4.4). In vitro, the iron-transferrin complex can be destabilised by attack upon the synergistic anion, or protonation and probably these mechanisms are involved in the release of iron from transferrin in vivo. There have been several reports demonstrating the release of (bi)carbonate from transferrin simultaneously with iron during iron uptake by reticulocytes (Egyed, 1973; Schulman et al., 1974). Furthermore replacement of (bi)carbonate in iron-transferrin complexes with oxalate

leads to a reduced rate of iron uptake (Egyed, 1973; Williams & Woodworth, 1973). These results are consistent with the hypothesis that the iron-release from transferrin involves the initial attack of the anion. However, the results do not prove the hypothesis as whatever mechanisms are involved in the release process, the anion will be released as well.

There is much evidence, however, that iron-release from transferrin may result from protonation of the iron-transferrin complex. The reaction of iron with transferrin involves the displacement of protons from the complex, and since this reaction is reversible, iron-release may involve addition of hydrogen ions. Evidence for this comes from the observation that the rate of iron release from transferrin is directly correlated to hydrogen-ion concentration and that iron is released from the transferrin-receptor complex only at low pH such as 4.8 - 5.5 (Dautry-Varsat, 1983; et al. Ciechanover et al., 1983; Klausner et al., 1983; Morgan, 1983). Further evidence for the importance of protonation in the release comes from studies in which treatment of cells with  $\text{NH}_4\text{Cl}$ , which neutralizes intracellular acidic compartments, blocks segregation of iron from transferrin after endocytosis while the reagent does not affect internalisation of transferrin (Ciechanover et al., 1983; Harding & Stahl, 1983; Loh, 1983; Rao et al., 1983). Since  $\text{NH}_4\text{Cl}$ , by causing an increase in intravacuolar pH, inhibits release of iron from transferrin, it seems reasonable that removal of iron may take place within the endocytic vacuole. The iron is released

when the pH is reduced by the action of a proton pump, which would account for the requirement for metabolic energy during the iron-uptake process. Alternatively, a lysosome may fuse with the endocytic vacuole thus reducing the intravacuolar pH to about 4.5 (Ohkuma & Poole, 1978). The apotransferrin-receptor complex which is stable at pH 4.8 (Klaunser et al., 1983) could escape digestion, and be recycled back to the exterior.

Agents such as pyrophosphates and organic phosphates have also been implicated in the removal of iron from transferrin in vitro (Coward et al., 1983) and it may be that ATP acts both as a source of energy and competing chelator (Morgan, 1979).

Literature data concerning functional differences between the two iron-binding sites have been controversial. It has been suggested that iron from one site is preferentially taken up by cells. Fletcher & Huehns (1967) reported that transferrin in the plasma preferentially delivers iron to immature red cells from one site, and that the other site was orientated towards the hepatocytes and intestinal mucosal cells. Further experiments, though, revealed no functional difference between the sites in homologous systems (Harris & Aisen, 1975; Harris, 1977; Huebers et al., 1981b; Heaphy & Williams, 1982b; Young, 1982). For heterologous systems, reports have been contradictory, and some authors have shown functional differences between the sites (Princiotta & Zapolski, 1976) while others did not (Esparza & Brock, 1980 ; Huebers et al., 1981b). Generally, evidence

suggests that the two iron-binding sites of transferrin are functionally the same with respect to iron donation to cells, although studies both in vitro (Huebers et al., 1981b) and in vivo (Huebers et al., 1978; 1981a,b,c) revealed preferential utilization of iron from diferric transferrin.

The released iron probably enters the labile pool present in the cell (Jacobs, 1977) from which it can be taken up by mitochondria for haem synthesis. The mechanism by which iron is delivered to the mitochondria has not yet been elucidated, although transferrin has been implicated in the direct transport of iron to the mitochondria (Romslo, 1980). Intracellular iron transport, however, is beyond the scope of this study and further information can be obtained from the review of Romslo (1980).

#### 1.2.4.6. Release of apotransferrin

Little attention has been directed to the mechanism of return of iron-depleted transferrin from the cell to its surrounding medium. However, there are reasons to suppose that apotransferrin leaves the cell by exocytosis. The kinetics, metabolic requirements and response to many cellular poisons of transferrin release in reticulocytes are similar to those of iron uptake. The differential effects of pH on the binding of apotransferrin and diferric transferrin to the receptor probably play an important part in the recycling process. At acid pH in the intracellular vesicles apotransferrin retains its high affinity for receptors and remains bound to them as explained in Section 1.2.4.5. However, once carried to the cell surface,

apotransferrin would be readily exchanged with diferric (or monoferric) transferrin because of their higher affinity for the receptor at neutral pH of the cell exterior. Both transferrin and receptor recycle with little evidence of degradation. However, in fibroblasts some degradation of transferrin occurs probably as a result of fluid phase endocytosis (Octave et al., 1981).

In conclusion, although some details still remain to be worked out, it is now clear that transferrin acts as a specific iron donor to immature erythroid and proliferating cells. Uptake probably occurs by receptor-mediated endocytosis, a mechanism in which the transferrin molecule is conserved while iron is acquired by the cell.



### 1.3. EFFECTS OF IRON DEFICIENCY ON CELL-MEDIATED IMMUNE RESPONSES AND LYMPHOCYTE TRANSFORMATION

#### 1.3.1. General considerations

As previously described, transferrin is important because of its ability to transport iron from the sites of iron absorption and catabolism to cells. Apart from erythroid precursors which need iron for the synthesis of haemoglobin, fast dividing cells also may require iron to meet their increased need for the synthesis of metabolically important enzymes and cytochromes. The expansion of clones of lymphocytes, characterised by an increased cellular metabolic activity and cell division, is associated with increased expression of transferrin receptors (see 1.2.4.3), which probably reflects an increase in iron requirement. Inadequate delivery of iron, therefore, may prevent lymphocytes from proliferating at optimum rate. Evidence to support this concept comes from literature data, which indicate that iron deficiency can result in decreased DNA synthesis, which could impair the cell-mediated immune response.

#### 1.3.2. Iron deficiency

Iron deficiency is the most widespread nutritional deficiency recognized throughout the world. The deficiency state arises through a variable combination of inadequate dietary intake, increased metabolic requirements and blood loss. Three steps are identifiable in the development of iron deficiency (Dallman, 1981). The first is characterised

by depletion of iron stores. If the iron deficiency progresses, the second phase appears. This phase is characterised by decreased saturation of transferrin with iron, and increased total iron binding capacity (TIBC) of transferrin. Depletion of both iron stores and transport of iron inhibits production of metabolically active compounds requiring iron either as a constituent or as a cofactor. Such deficiencies may lead to systemic manifestations. The final stage in iron deficiency is characterised by hypochromic, microcytic anaemia.

#### 1.3.3. Cellular iron requirement

Iron is important for the activity of many enzymes (Wrigglesworth & Baum, 1980). Recently increased attention has been focused on the association of iron deficiency with inhibition of DNA synthesis and cell division. Hershko et al. (1970) observed a decrease in the nucleic acid content of bone marrow and also a marked drop in the rate of  $^3\text{H}$ -thymidine incorporation into DNA associated with chronic iron deficiency. Hoffbrand et al. (1976) offered evidence for a mechanism by which iron deficiency may reduce DNA synthesis. They observed that DFO produced an imbalance between DNA precursors and concluded that the removal of iron inhibited the iron-containing enzyme ribonucleotide reductase, since DFO inhibited the enzyme in vitro and the imbalance of deoxyribonucleotides paralleled the effects of hydroxyurea, a known inhibitor of reductase activity. Retardation of cellular and tissue development associated with iron deficiency, probably the

result of reduced DNA synthesis (Kochanowski & Sherman, 1982) has also been reported. Rothenbacher & Sherman (1980) observed that 18-day old rat pups from iron-deficient pregnant dams showed severe fatty degeneration of the liver and lesions in the spleen and thymus. In addition iron deficiency may impair the development of the central nervous system and cause behavioural changes, as indicated by low behavioural responses and low mental scores in young children (Seshadri et al., 1982) or low mental development scores in infants (Lozoff et al., 1982; Walter et al., 1983). The impaired development of the central nervous system in iron deficiency may be the result, in part, of decreased neuronal growth and myelinization due to impaired DNA synthesis and also decreased activity of an iron-dependent enzyme involved in neurotransmission (Leibel et al., 1979).

In conclusion, literature data indicate that DNA synthesis is impaired in iron deficiency. The reduced DNA synthesis in iron deficiency can be reflected in reduced cell-mediated immune responses in man and in animals.

#### 1.3.4. Iron deficiency and cell-mediated immunity

Since it is well documented that iron deficiency in Man is associated with increased incidence of infectious disease, attempts have been made to study the capacity of iron-deficient subjects to mount immune response. Cell-mediated immune responses, as assessed by in vivo delayed type hypersensitivity reactions to various antigens or in vitro by the proliferative response of lymphocytes, were impaired

in iron deficiency, both in Man and in animals. Joynson et al. (1972) studied cell-mediated immunity in adults with iron deficiency anaemia. Delayed hypersensitivity and lymphocyte transformation in response to purified protein derivative (PPD) of Mycobacterium tuberculosis and Candida antigen were impaired in iron deficient anaemic adults compared with a control group. In addition, the production of macrophage-migration inhibition factor was significantly reduced when lymphocytes were stimulated with Candida but only slightly reduced when PPD was used. Macdougall et al. (1975) evaluated CMI responses in children with latent iron deficiency, iron deficiency anaemia and healthy controls. Delayed type hypersensitivity reactions in vivo and DNA synthesis in vitro by stimulated lymphocytes were impaired in the anaemic and latent iron-deficient children. Similar observations have been reported by others (Chandra & Saraya, 1975), who in addition, showed that the proportion of T-lymphocytes was slightly but significantly reduced in the iron-deficient group in comparison with the control group. Fletcher et al. (1975) found lymphopaenia and depressed lymphocyte mitogenic responses in iron-deficient subjects which returned to normal after iron therapy. Bhaskaram & Reddy (1975) reported irreversible in vitro reduction in the proliferation of lymphocytes isolated from iron-deficient patients. They also reported decreased T-cell numbers in the iron-deficient group which increased after correction of the iron status. Depressed lymphocyte function as assessed by DTH test in response to PHA and in vitro by leucocyte migration inhibition

tests have also been reported in association with iron deficiency anaemia (Talwalkar et al., 1982). Morphological abnormalities in lymphocyte mitochondria in iron-deficient patients have also been observed. Swelling, vacuolation and rupture of the cristae of peripheral blood lymphocytes in iron-deficient anaemic patients have been reported (Jarvis & Jacobs, 1974). Similar mitochondrion abnormalities in iron-deficient anaemic subjects have been observed by Jiménez et al. (1982), who, in addition, showed that the mitochondrion modifications were proportional to the degree of iron deficiency. Abnormalities in mitochondria in lymphocytes in iron deficiency may result in a defective activity of the mitochondria which then may affect cellular metabolic activity, resulting in reduced cell proliferation. However, an association between abnormalities in lymphocyte mitochondria and cell proliferation has not yet been demonstrated. Taken together these reports indicate that CMI responses are impaired in iron deficiency. However normal CMI responses in iron deficiency have also been reported. The number of T-cells and the proliferation of peripheral blood lymphocytes in response to mitogens in iron deficiency were not statistically different from the values obtained from control groups (Van Heerden et al., 1981). Furthermore, normal in vitro proliferation of lymphocytes isolated from iron-deficient (Gross et al., 1975; Srikanthia et al., 1976; Krantman et al., 1982) or iron-deficient anaemic patients (Kulapongs et al., 1974; Sawitsky et al., 1976; Gupta et al., 1982) have also been reported.

There are, therefore, controversial literature data on whether iron deficiency can result in depressed lymphocyte transformation and CMI responses in Man, which, in turn, may result in increased susceptibility to infections. Firm conclusions, therefore, cannot be drawn regarding cell-mediated immunity in patients with iron deficiency. Infections characteristically seen in immunodeficient states due to severe defects of cell-mediated immunity such as disseminated viral or fungal diseases, have not yet been reported in association with iron deficiency. Certainly patients with iron deficiency may escape such infections, but it is then difficult to implicate the deficiency of iron as a causative mechanism for any infection that may occur. Oral candidiasis (Fletcher et al., 1975) and chronic mucocutaneous candidiasis, (Higgs & Wells, 1972) in association with iron deficiency have been reported before, but candidiasis is classically found in patients with impaired cell-mediated immunity. However, other factors may also predispose the iron-deficient patients to candidiasis, since not all iron-deficient patients with documented decreased CMI responses have candidiasis (Higgs & Wells, 1972; Fletcher et al., 1975). On the contrary, leukaemic patients which have increased plasma iron and increased transferrin saturation also have problems with mucocutaneous candidiasis. Obviously, factors associated with iron balance but unrelated to cell-mediated immunity may also be important. In animal models, though, it has been shown that impaired host responses associated with iron deficiency may contribute to increased susceptibility

to gut parasites. Thus Duncombe et al. (1979) have shown that iron deficiency suppressed resistance to reinfection with Nippostrongylus brasiliensis in the rat and that iron repletion effectively restored the protective immunity. Other defects of cell-mediated immunity in iron deficiency in animals have been reported. Kuvibidila et al. (1982) reported a decreased proportion of T-cells in the spleens of iron-deficient mice. Decreased numbers of T-cells may result in a reduced proliferative response of the lymphocytes in vivo. Impaired in vitro blastogenic response of splenic lymphocytes isolated from iron-deficient mice has also been reported (Kuvibidila et al., 1983a,b), which was corrected by iron supplementation, showing that the defect in iron deficiency can be corrected after iron therapy. In another study, delayed cutaneous hypersensitivity of mice to dinitrofluorobenzene was reduced in association with iron deficiency (Kuvibidila et al., 1981), this being the result of reduced DNA synthesis as assessed by incorporation of labelled DNA-precursors into the sensitised area. Furthermore, the DTH reaction returned to normal on administration of iron dextran prior to the recall dose of dinitrofluorobenzene. Taken together these observations indicate that in iron deficiency some function of lymphocytes associated with the ability of cells to proliferate is defective, and that this defect is corrected after iron therapy. In contrast to the above findings, Soyano et al. (1982) demonstrated increased proliferation of splenic lymphocytes in response to optimum doses of mitogen, but a decreased response of

thymic lymphocytes isolated from iron deficient rats. Their results might be explained by either an alteration or imbalance of the population of cells in various lymphoid compartments, or alteration in the migration pattern of lymphocytes due to iron deficiency.

In conclusion, literature data on the effect of iron deficiency on cell transformation showed that in vivo the proliferative response of lymphocytes is impaired both in Man and in animal. However, data arising from the in vitro studies are contradictory and some investigators found reduced mitogenic or antigenic responses of lymphocytes while others reported normal proliferation. The exact effect of iron deficiency on cell proliferation, therefore, remains to be elucidated, and forms the subject of part of the work reported in this thesis.



CHAPTER 2  
PRELIMINARY EXPERIMENTS

## 2.1 INTRODUCTION

The work presented in this thesis involved the study of the effect of iron and transferrin on mouse lymph node cells in vitro. The proliferative response was estimated by measuring the degree of incorporation by Con A-stimulated lymphocytes of labelled thymidine into newly synthesized DNA.

Since incorporation of labelled thymidine into the cells may not reflect proliferation, parallel morphological evaluation or autoradiography may be necessary to assist in the estimation of proliferative responses. However, parallel studies using autoradiography as well as estimation of DNA synthesis by measuring the degree of labelled thymidine incorporation indicates that it is the transformed cells which incorporate the label (MacKinney et al., 1962).

Furthermore, lymphocyte proliferation in vitro measured by the technique of incorporation of labelled thymidine has been used for a long time to provide insight into the pathogenesis of disease involving immune mechanisms (Aisenberg, 1965; Bjune et al., 1976; Bjune, 1979), and also to study the effects on cell-mediated immunity of iron deficiency with or without anaemia (Joynson et al., 1972; Bhaskarom & Reddy, 1975; Fletcher et al., 1975; Gross et al., 1975; Macdougall et al., 1975; Srikantia et al., 1976; Van Heerden et al., 1981; Krantman et al., 1982; Soyano et al., 1982; Kuvibidila et al., 1983a,b ). Additionally, the technique has been used to investigate the effect of certain drugs on

cell-mediated immune responses (Bonnet et al., 1984; Payan et al., 1984). The study of the effects of growth factors involved in T-cell proliferation (Phillips & Azari, 1975; Andersson et al., 1979; Coutinho et al., 1979; Brock, 1981; Novogrodsky et al., 1982; Lipkowitz et al., 1984; Malek & Shevach, 1984), or accessory-cell cooperation (Smith et al., 1980b; Sunshine et al., 1980; Fitzgerald et al., 1983) has also been achieved by measuring proliferation as the degree of incorporation of labelled thymidine into newly synthesized DNA.

In the present investigation, the experimental technique, in particular the use of serum-free medium to culture the cells, was developed by Brock (1981) using 1 ml cultures in test tubes, and since the technique needed to be adapted to a microculture method, some preliminary work was essential in order to find the exact conditions to perform the experiments.

One of the important factors which may affect the estimation of the proliferative response of the lymphocytes is the period for which the cells are cultured before proliferation is measured. At certain times, measurement of incorporation of labelled thymidine into newly synthesized DNA may not reflect the actual degree of cell-proliferation as some cells may undergo transformation without having entered the S phase of the cell cycle. It was therefore important to find the time for which lymph node cells should be cultured in order to get maximum thymidine incorporation.

The degree of response to ConA of mouse lymph node cells in serum-free and serum-containing media was therefore estimated at different time intervals. Furthermore, since the degree of lymphocyte proliferation after mitogenic stimulation depends on the amount of the mitogen used (Stobo et al., 1972; Ling & Kay, 1975; Wang et al., 1976), it was also essential to find the concentration of ConA which induced maximum proliferation. As previous work in this laboratory (Brock, personal communications) showed that in test-tube cultures optimum transformation of mouse lymph node cells in serum-free and serum-containing culture medium was achieved at concentrations of ConA of 1 and 8  $\mu\text{g/ml}$ , respectively, in the present investigation a range of values of 0.025 - 4  $\mu\text{g/ml}$  for serum-free and 1 - 16  $\mu\text{g/ml}$  for serum-containing medium was used.

It has already been established that when lymphocytes transform in serum-free conditions, their response depends on the availability of transferrin and iron (Phillips & Azari, 1975; Brock, 1981; Brock & Rankin, 1981). Since different saturations of transferrin with iron have different effects on the proliferation of lymphocytes (Brock, 1981), it was essential in the present work, to find the saturation of transferrin with iron which induced a good proliferative response in order to use it in the subsequent experiments as a standard positive control. Additionally, as large amounts of transferrin were needed to complete the studies, it was important to determine whether human transferrin

which has the advantage of being commercially available in a highly purified state, could be substituted for the homologous mouse transferrin in the in vitro mouse lymphocyte cultures.

Lymph node cells contain a small percentage of cells other than lymphocytes such as monocytes or macrophages. The degree of incorporation of labelled thymidine into new DNA might, therefore, also reflect replication of these adherent cells. Thus, an experiment was performed in which the incorporation of labelled thymidine by lymph node cells was studied in the presence and absence of adherent cells.

## 2.2 MATERIALS

2.2.1. Animals. Unless otherwise stated, female C<sub>3</sub>H/Bi inbred mice, 12-17 weeks old were used throughout, and obtained from the departmental animal house stock.

2.2.2. Reagents. When necessary, media were prepared in iron free apparatus (see 2.3.1.2).

2.2.2.1. Phosphate buffered saline (PBS). This consisted of NaCl (8 g/l), KCl (0.2 g/l), Na<sub>2</sub>HPO<sub>4</sub> (1.15 g/l) and KH<sub>2</sub>PO<sub>4</sub> (0.2 g/l) (Analar grade, BDH Chemicals Ltd., Poole, Dorset, England). Solutions were made in de-ionised distilled water (d-DW) and when appropriate sterilised by filtration.

2.2.2.2. Ferric nitrilotriacetate solution Fe(NTA), 10 mM.

This solution was prepared by mixing one volume of a freshly prepared solution of FeCl<sub>3</sub> (20 mM; BDH) and one volume of NaNTA (80 mM; pH 7; Sigma Chemical Co., Poole, Dorset, England). The FeCl<sub>3</sub> was added to the NaNTA dropwise with constant stirring, adjusting the pH to 5 as required with 1 M NaOH to prevent the NTA-free acid precipitating. The final pH was 5.

2.2.2.3. Scintillation fluid. This solution was prepared by mixing one volume of Triton-X-100 to two volumes of a solution of 2,5 diphenyloxazole (5 g/l) and 1,4-di(2(5-phenyloxazolyl)benzene) (0.3 g/l) in toluene at 56°C (all chemicals obtained from BDH).

2.2.2.4. Concanavalin A solution. Concanavalin A was obtained from Miles Laboratories, Stoke Poges, Slough, Bucks, England

and solutions of the appropriate concentrations (see 2.4.2) were prepared in PBS, sterilised by filtration and stored in aliquots of 100  $\mu$ l at  $-20^{\circ}\text{C}$ . Each aliquot was used once only.

2.2.2.5. Human serum albumin solution. A solution of 1% (w/v) human serum albumin (HSA; Behringwerke, Hoechst, Hounslow, Middlesex, England) was prepared in RPMI-1640 culture medium (Flow Laboratories, Irvine, Strathclyde, Scotland). The solution was sterilised by filtration and stored at  $4^{\circ}\text{C}$ . The albumin contained no detectable transferrin (J.H. Brock, personal communications).

2.2.2.6. Human apotransferrin solution. Iron free (apo-) transferrin was obtained from Behringwerke. A stock solution (10 mg/ml) was prepared in PBS containing 1% (w/v)  $\text{NaHCO}_3$  (Analar grade, BDH) to ensure iron binding, sterilised by filtration and stored at  $4^{\circ}\text{C}$ . It was used within a month. For 20 or 25% iron saturated transferrin preparations, 3.0 or 3.75  $\mu\text{g/ml}$ , respectively, of iron as ferric nitrilotriacetate complex (see 2.2.2.2) was added. For other saturations of transferrin with iron, the appropriate amount of  $\text{Fe(NTA)}$  was used.

2.2.2.7. Standard culture medium. The standard culture medium consisted of RPMI-1640 (without Hepes; Flow) supplemented with penicillin (100 Iu/ml) and streptomycin (100  $\mu\text{g/ml}$ ) (Flow) and 0.3 mg/ml L-glutamine (BDH). To this 2-mercapto-ethanol (2-Me; final concentration 50  $\mu\text{M}$ ; BDH) and 10% final concentration of foetal calf serum (FCS; Gibco, Paisley, Scotland) were added. For serum-free conditions, HSA (1 mg/ml

final concentration) and human transferrin (usually 50 µg/ml final concentration and 30% saturated with iron including the iron present in the culture medium; see section 2.4.3) were used instead of FCS.

## 2.3 METHODS

### 2.3.1. Estimation of iron

#### 2.3.1.1. Estimation of iron in the culture medium.

The iron content of the serum-free culture medium (see 2.2.2.7) was determined by Dr D. Halls, Department of Biochemistry, Glasgow Royal Infirmary, Scotland, using carbon furnace atomic absorption spectroscopy. Two different batches of RPMI-1640 without Hepes culture medium were used during this work. The iron content of the first batch was 12.5 ng/ml and that of the second 5 ng/ml. These levels of iron were enough to saturate 8.9% and 3.4%, respectively of the added transferrin, and these figures were rounded off to 10% and 5% respectively, in subsequent experiments.

#### 2.3.1.2. Estimation of the iron contamination of plasticware used to prepare media or culture cells.

Iron free plastic bijou, test tubes and pipette tips were used throughout this work and were selected from a number of items purchased from different suppliers after estimating their contamination with iron spectrophotometrically, using the reagents prepared for serum iron estimation (see Section 3.2.3). The reagents were left in contact with the item for 30 min and an aliquot was then aspirated and the absorbance was measured against a reagent blank in a



1 cm cuvette. Usually ten articles of the same kind were tested, and the iron-free were selected on the basis of low absorbance (zero - 0.002).

The following items were found to be iron-free and used in the subsequent experiments:

1. Sterile 7 ml plastic bijou (Sterilin, Teddington, Middlesex, England).
2. Sterile 20 ml plastic universal bottles (Sterilin; not tested but informed of suitability by Dr D. Halls).
3. Plastic 10 ml conical bottomed test tubes (Sterilin).
4. Polypropylene test tubes (Falcon, Becton-Dickinson, Oxnard Ca, U.S.A.).
5. Plastic 50 ml tissue culture flasks (Gibco).
6. Sterile 96V - well microtitre tissue culture plates (Linbro Chemical Co., New Haven, Conn., U.S.A.).
7. Plastic 1.5 ml Eppendorf reaction vials (Anderman & Co. Ltd., East Molesey, Surrey, England).
8. Treff yellow plastic pipette tips (0.200  $\mu$ l; Scotlab Instruments Ltd., Carlisle, Lanarks, Scotland).
9. Volac blue plastic pipette tips (0-1000  $\mu$ l; A & J. Beveridge Ltd., Edinburgh, Scotland).

Glassware was rendered iron-free by washing in 10% HCl solution for 10 h and then rinsing in d-DW three times.

#### 2.3.2. Preparation of cell suspension and culture conditions.

Cell suspensions were prepared from the peripheral (brachial, axillary and inguinal) and mesenteric lymph nodes of the mice. The lymph nodes were removed from freshly

killed mice under sterile conditions and the cells were teased into RPMI-1640 with Hepes (Gibco) culture medium supplemented with penicillin, streptomycin and L-glutamine as described in Section 2.2.2.7. The cell suspension was mixed well, transferred into a sterile universal bottle and left to stand for 1-2 min to allow any pieces of tissue to settle to the bottom. The cell suspension was then carefully aspirated into a clean universal bottle and the cells were washed once at 1200 rpm for 45-60 sec in the culture medium. The suspension contained 90-95% lymphocytes as demonstrated by Leishman stain and were 85-95% viable as measured by eosin exclusion. The cells were finally suspended in the culture medium (see 2.2.2.7) at a concentration of  $2.3 \times 10^6$  viable cells/ml and, unless otherwise stated, were cultured in conical bottomed microtitre culture plates (see 2.3.1.2), each well containing  $2 \times 10^5$  cells and ConA at the appropriate concentration (see 2.4.2) in a total volume of 100  $\mu$ l. The importance of adding ConA last should be noted. Preliminary experiments showed that the addition of ConA to the medium before adding the cells prevented transformation particularly in serum-free medium. The cells were cultured at 37°C in an atmosphere of 10% CO<sub>2</sub>, 90% air using a New Brunswick humidified CO<sub>2</sub> incubator, for 2-3 days, as required. Proliferative responses were assayed by pulsing cells for 4 h with 50  $\mu$ l/well of <sup>14</sup>C-thymidine containing 12.5 nCi (Specific activity 50-60 mCi/mmol; Amersham International, England) before the cells were

harvested on a Skatron cell harvester (Flow). The cell-associated radioactivity was measured for 5 min in a Packard liquid scintillation counter (Model 3320). Unless otherwise stated, the cultures were set up in heptuplicates with quadruplicate ConA-negative controls.

### 2.3.3. Preparation of mouse serum transferrin

Mouse transferrin was prepared from pooled mouse serum using the method described by Brock et al. (1976) for the preparation of bovine transferrin except that the initial step of rivanol (6,9-di-amino-2-ethoxy acridine lactate) precipitation step was omitted. Mouse serum was prepared by centrifugation of clotted blood, and after aspiration of the serum enough iron from  $\text{Fe(NTA)}$  (see 2.2.2.2) was added to fully saturate the transferrin. The  $\gamma$ -globulin of the serum was precipitated at  $5^{\circ}\text{C}$  for 24 h with  $2\text{M}(\text{NH}_4)_2\text{SO}_4$  (BDH) and the supernatant containing the transferrin was aspirated after centrifugation in a MSE-high speed-18 centrifuge for 20 min, at 12,000 rpm. Crude transferrin was precipitated from this solution at  $5^{\circ}\text{C}$  for 24 h by increasing the  $(\text{NH}_4)_2\text{SO}_4$  concentration to 2.8 M and recovered by further centrifugation. The precipitate was dissolved in distilled water (DW) and dialysed against 0.05 M Tris-(hydroxymethyl) methylamine (Tris) pH 7.6 for 20-24 h. Further purification of the transferrin preparation was achieved by chromatography on DEAE-Sephadex A50 (Pharmacia, Uppsala, Sweden). The column was equilibrated with 0.05 M  $\text{NaCl}/0.05$  M Tris, pH 7.6, and the transferrin was eluted

first with equilibrating buffer and then by stepwise increase of NaCl concentration from 0.05 to 0.10 M in 0.05 M Tris, pH 7.6. Transferrin eluted at 0.10 M NaCl but still contained a contaminant (presumed to be haemopexin) which was removed by chromatography on SP-Sephadex C50 (Pharmacia) equilibrated with citrate buffer, 0.02 M, pH 5.1, and eluted with citrate buffer by a stepwise increase of the pH from 5.1 to 5.55. The transferrin was eluted at pH 5.55. Removal of any remaining iron was achieved by dialysis against 0.02 M sodium citrate pH 5.1. The transferrin solution thus obtained was concentrated by ultrafiltration and the purity was tested by zonal cellulose acetate electrophoresis in barbitone buffer (pH 8.6) and mouse transferrin preparation as a standard. The barbitone buffer consisted of Tris (4.8 g/l), barbitone (2.06 g/l) and sodium barbitone (8.13 g/l). The samples were applied at the cathode and run under a constant current (20 mA) for 1 h. The concentration of the transferrin in the solution was estimated spectrophotometrically by measuring the absorbance at 280 nm and using the extinction coefficient value;  $E_{1\text{cm}}^{1\%} = 11.2$  for human transferrin (Feeney & Komatsu, 1966). Finally the preparation was dialysed against three changes of DW at 5°C for 24 h and freeze-dried.

#### 2.3.4. Leishman stain

About  $6 \times 10^5$  cells were deposited on to clean slides in a Shandon cytocentrifuge at 1200 r.p.m. for 90 sec. The preparations were air dried and then fixed and stained by immersing the slides in undiluted Leishman stain for 2 min and then in diluted stain (1 in 3 in DW) for 10 min. The

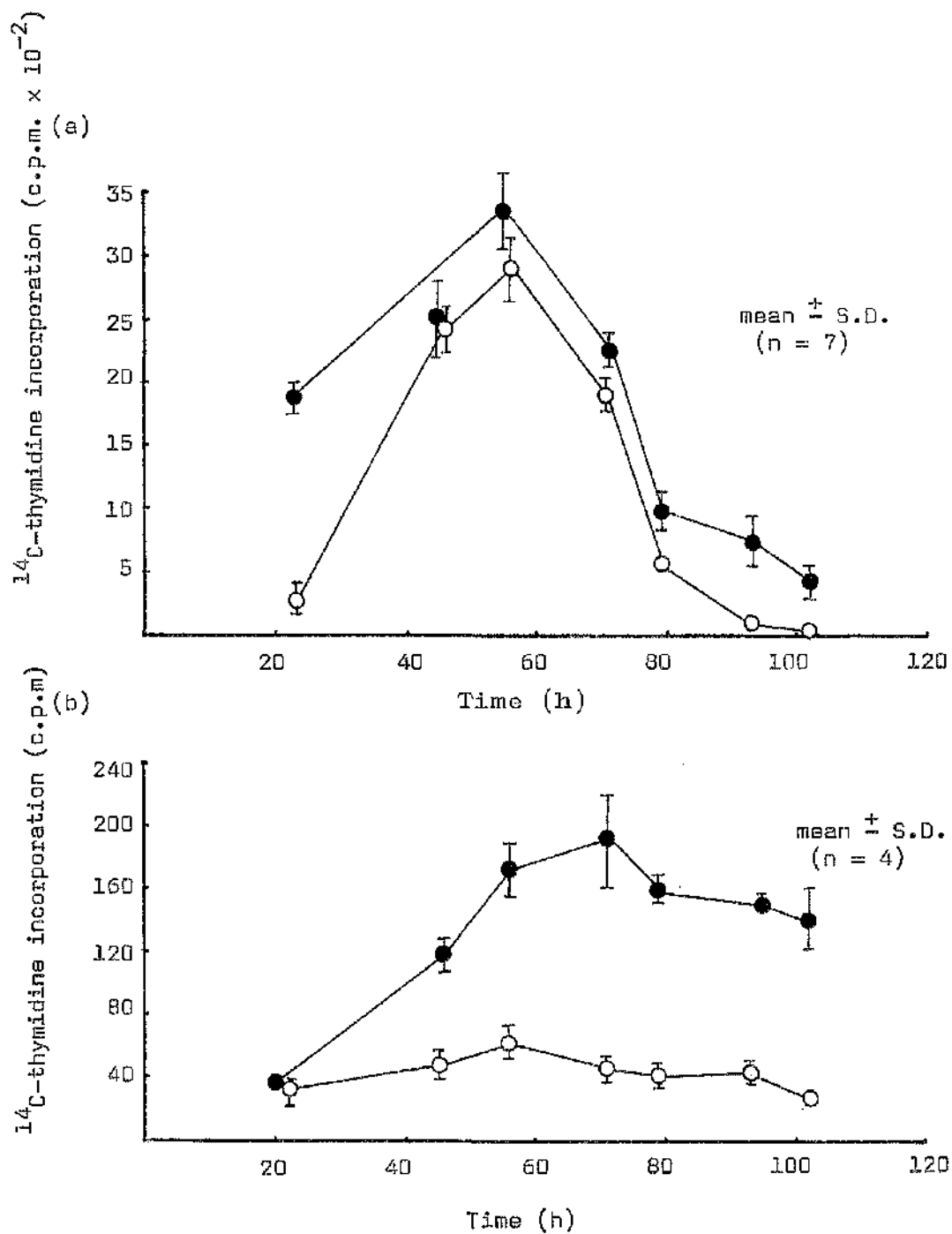
slides were then washed in tap water, air-dried and examined in a Leitz-Wetzlar light microscope (x 200). The number of large blast like cells and total cell counts in different fields taken at random were obtained and the percentage of blasts was calculated. About 300-350 cells were counted each time.

## 2.4 RESULTS

### 2.4.1. Time-course of the proliferative response of mouse lymph node lymphocytes to concanavalin A in vitro

Mouse lymph node cells were cultured in serum-free and serum-containing medium as described in Section 2.3.2. At different time intervals cells were pulsed, harvested and their radioactivity was measured (see 2.3.2). Controls without ConA were also included. The maximum proliferative response in both media was obtained between 46-71 h with the peak at 56 h (Fig. 2a), and therefore, in the subsequent experiments, the cells were cultured for 55-69 h. Although in this experiment the degree of transformation of the cells when cultured with ConA in medium containing serum was not very much higher than the response when serum-free medium was used (Fig. 2a), serum-containing medium sometimes induced better transformation than the serum-free conditions (Fig. 3, and other preliminary experiments not reported here). This was probably due to the fact that the serum-containing medium contains nutrients and growth-promoting factors other than albumin and transferrin, hence the enhanced proliferation of the lymphocytes. In the absence of ConA the degree of transformation in both media was very low (Fig. 2b). However, there was some transformation observed which might have been the result of non-specific stimulation of some lymphocytes due to a few mesenteric lymph node cells having been antigenically stimulated prior to culturing. Furthermore, the background transformation of lymphocytes in serum-containing medium was very much higher than when the cells were cultured in serum-free conditions.

Figure 2: In vitro transformation of mouse lymphocytes in serum-containing (■ - ■) and serum-free (○ - ○) medium.



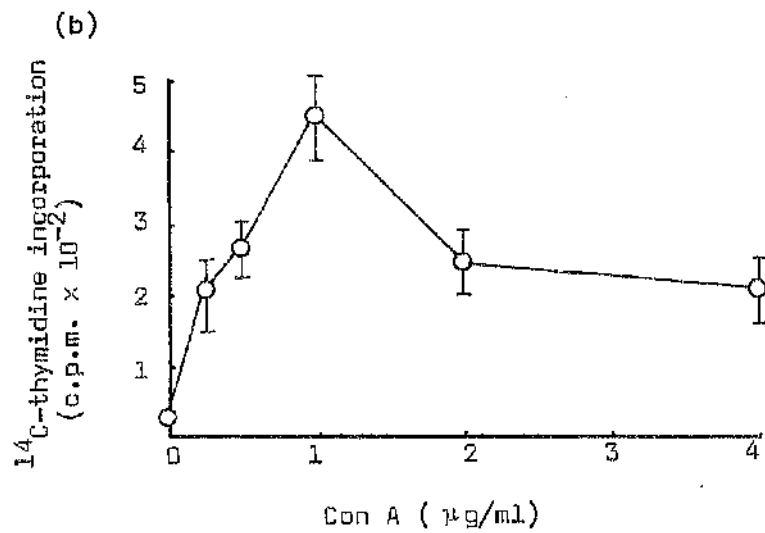
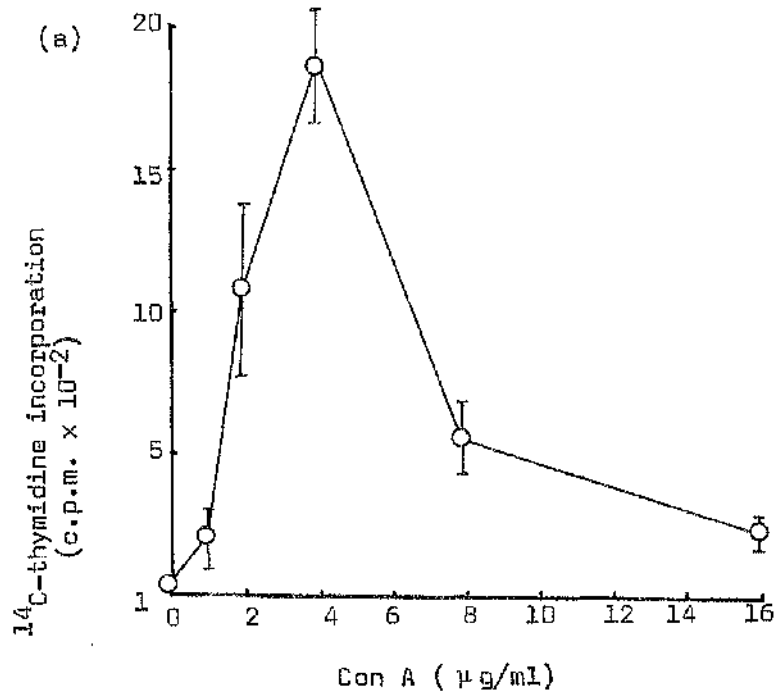
- (a) Cells cultured in medium containing concanavalin A (4  $\mu\text{g}/\text{ml}$  for serum-containing medium, 1  $\mu\text{g}/\text{ml}$  for serum-free medium)
- (b) Cells cultured without concanavalin A.

2.4.2. The effect of different concentrations of concanavalin A on the proliferative response of mouse lymph node cells

Lymph node cell suspensions were prepared as described in Section 2.3.2. The cells were cultured in serum-free medium (see 2.2.2.7) containing 0.25, 0.5, 1, 2 and 4  $\mu\text{g/ml}$  of ConA, or in serum-containing medium (see 2.2.2.7) containing 1, 2, 4, 8 and 16  $\mu\text{g/ml}$  of ConA. The cells were cultured for 61 h and proliferative responses were assayed as described in Section 2.3.2. Two different titration experiments were performed using different batches of RPMI-1640 medium and ConA. In the first experiment, culture medium containing 12.5  $\mu\text{g iron/ml}$  was used. When a new batch of ConA was required, it was titrated using a new batch of culture medium in which the endogenous iron was 5  $\text{ng/ml}$ . Figure (3a,b) shows the responses of lymphocytes to ConA in serum-containing and serum-free medium, respectively using the first batch of ConA. The concentrations of ConA which induced optimum transformation were 4 and 1  $\mu\text{g/ml}$ , respectively. However, when the new batch of ConA was used, maximum transformation was obtained at concentrations of 2 and 0.5  $\mu\text{g/ml}$  for serum-containing or serum-free medium, respectively (Fig. 4). The reason that higher concentrations of ConA were needed to induce optimum transformation in serum-containing than in serum-free medium was probably that some of the ConA binds to serum proteins in the medium. In the subsequent experiments the concentrations of ConA used were 1 or 0.5  $\mu\text{g/ml}$  for serum-free medium and 4 or 2  $\mu\text{g/ml}$  for serum-containing medium for the first and second batches of ConA, respectively.

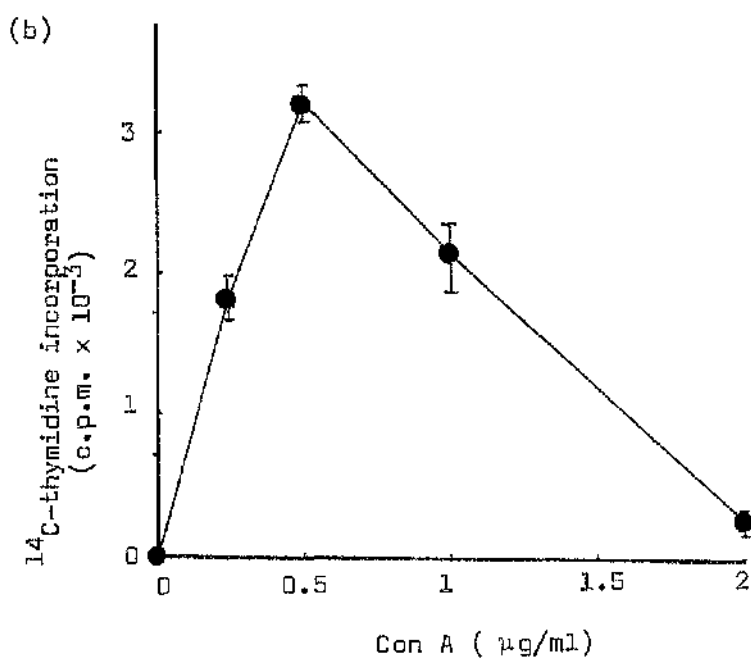
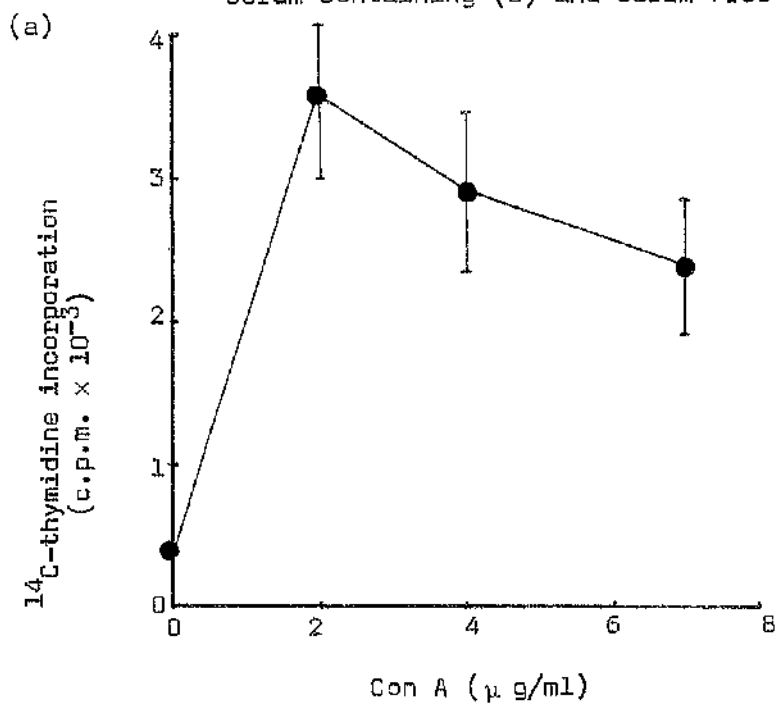


Figure 3: The effect of concanavalin A (batch 1) on the in vitro transformation of mouse lymphocytes in serum-containing (a) and serum-free (b) medium.



Mean  $\pm$  S.D. (n = 7)

**Figure 4:** The effect of concanavalin A (batch 2) on the in vitro transformation of mouse lymphocytes in serum-containing (a) and serum-free medium (b).

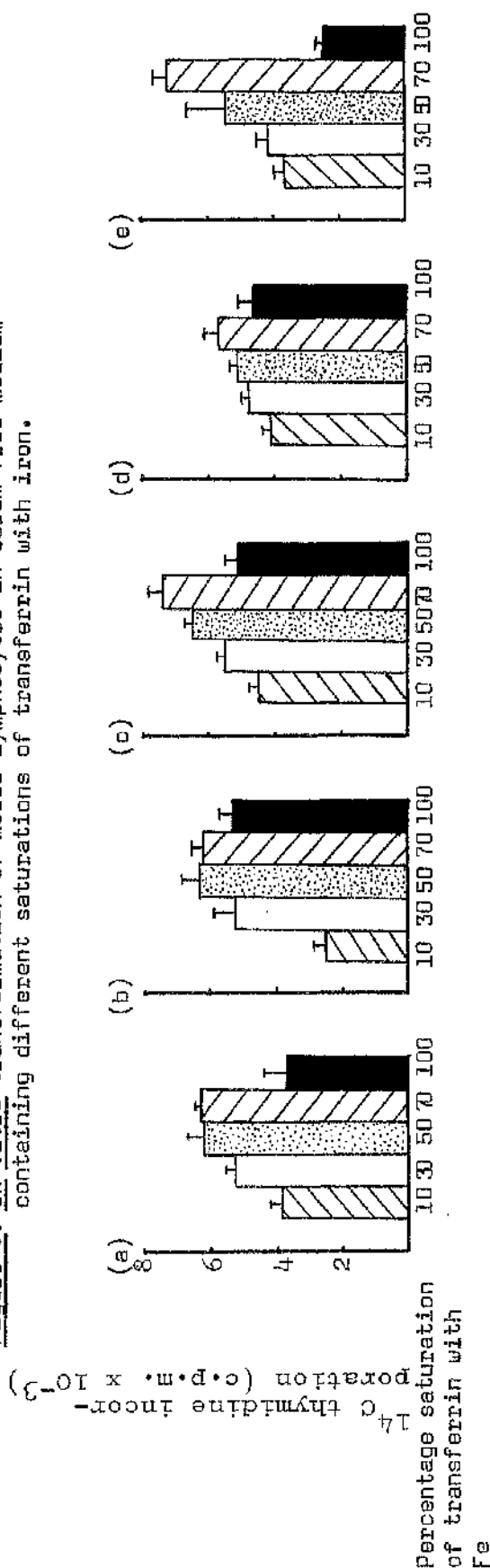


Mean ± S.D. (n = 7)

2.4.3. The response to concanavalin A of mouse lymph node cells in serum-free media containing different saturations with iron

Lymph node cells were cultured with ConA 1  $\mu$ g/ml (see 2.4.2) in serum-free medium containing transferrin of total saturation with iron of 10, 30, 50, 70 and 100 per cent (see 2.2.2.6). Negative controls without ConA were also included. The cultures were set up in quadruplicate with duplicate ConA-negative controls. Two-way analysis of variance (Two-way ANOVA) of all observations (excluding those when 100%-iron-saturated transferrin was used) showed that in medium containing 10%-iron-saturated transferrin the proliferative response of the lymphocytes was significantly lower than when transferrin 30 to 70 per cent saturated with iron was used (Fig. 5). In subsequent experiments, therefore, the effect of restriction of iron on lymphocyte transformation was investigated by using 10%-iron-saturated transferrin in the culture medium. The degree of transformation of lymphocytes in medium containing 100%-iron-saturated transferrin was also very much reduced. This suggests that at very high saturation of transferrin with iron, the iron may be toxic to the cells. Nevertheless, in subsequent experiments the cells were cultured in medium containing 30%-iron-saturated transferrin for a positive control response. This saturation of transferrin was chosen for the reason that it may be more appropriate to conditions in a responding lymph node (see Discussion, p.78 ). Furthermore, a difference

Figure 5: In vitro transformation of mouse lymphocytes in serum-free medium containing different saturations of transferrin with iron.



Five experiments performed (a,b,c,d,e). Each block represents mean counts + S.D. (n = 4). Mean counts of negative controls (n = 2)  $\leq$  100 c.p.m.

Two-way ANOVA of results of five experiments using 10,30,50 or 70% FeTf: day-to-day variation and effect of iron-saturation of transferrin, highly significant ( $F = 7.47$ ,  $p < 0.001$ ;  $F = 56.30$ ,  $p < 0.001$ ; respectively). Interaction: Tfx experiment: not significant.

in lymphocyte transformation-promoting activity between 10 and 30 per cent iron-saturated transferrin was consistently observed in this set of experiments as well as in other experiments (see Chapter 3).

2.4.4. Effect of mouse and human transferrins on the response of mouse lymph node cells to concanavalin A

Mouse lymph node cells were cultured as described before (see 2.3.2) in serum-free medium containing ConA (0.5 µg/ml; see 2.4.2) and 30%-iron-saturated human or mouse transferrin. Experiments using 10%-iron-saturated transferrins were also included. The degree of transformation of lymphocytes in medium containing 30%-iron-saturated mouse transferrin was not statistically different from the response in medium containing 30%-iron-saturated human transferrin (Table 1). Further, the mitogenic response of lymphocytes in medium containing 10%-iron-saturated mouse or human transferrin did not differ from each other, and were reduced compared with the responses when 30%-iron-saturated transferrins were used. These results indicate that human transferrin does not differ from mouse transferrin in its ability to promote lymphocyte proliferation and that human transferrin can be substituted for mouse for the in vitro lymphocyte cultures in serum-free conditions.

2.4.5. Effect of adherent cells on the transformation of mouse lymph node cells

Mouse lymph node cells ( $2 \times 10^6$  viable cells/ml) were cultured in serum-free or serum-containing medium in flat-

Table 1. The effect of mouse and human transferrins on the in vitro transformation of mouse lymphocytes in serum-free medium.

| <sup>14</sup> C-thymidine incorporation (c.p.m.) |                                  |                      |            |
|--|----------------------------------|----------------------|------------|
| Saturation of transferrin with iron:             | Mouse transferrin I <sup>+</sup> | Human transferrin II |            |
| 30%  |                                  |                      |            |
| + ConA*  | 2970 ± 498                       | 2940 ± 140           | 2809 ± 425 |
| - ConA   | 62 ± 7                           | 57 ± 5               | 57 ± 26    |
| 10%  |                                  |                      |            |
| + ConA*  | 1471 ± 443                       | 1918 ± 112           | 1222 ± 482 |
| - ConA   | 36 ± 7                           | 92 ± 22              | 35 ± 2     |
|  |                                  |                      | 2855 ± 131 |
|  |                                  |                      | 96 ± 37    |
|  |                                  |                      | 1725 ± 180 |
|  |                                  |                      | 73 ± 9     |

\* p > 0.1 in all cases. Mean ± S.D. + ConA, n = 7; -ConA, n = 4.

+ Two experiments were performed, I & II.

bottomed 24-well plates (Linbro Chemical Co.), each well containing 1 ml of cell suspension and ConA at 1 or 4  $\mu\text{g/ml}$  (see 2.4.2), respectively. The cells were cultured as described before (see 2.3.2) for 65 h before being pulsed with 50 nCi of  $^{14}\text{C}$ -thymidine for 4 h. However, before the cells of each well were pulsed, they were mixed and the medium containing the cells was transferred to a clean well in the plate in order to separate the adherent cells from the lymphocytes. Controls consisting of cells cultured but not transferred before being pulsed, as well as negative controls without ConA were included. The cultures were set up in quadruplicate with duplicate ConA-negative controls. Incorporation of  $^{14}\text{C}$ -thymidine into cells which were transferred into clean wells before being pulsed with the labelled thymidine was the same as cells which were not transferred (Table 2), in both serum-containing or serum-free medium. Microscopic observations with a Nikon phase contrast microscope (x200) of the wells from which cell suspensions were removed revealed macrophage-like cells adherent to the bottom of the wells. These results indicate that the cell-associated radioactivity was due to activity of lymphocytes and not due to that of monocytes or macrophages.

The degree of transformation in the absence of ConA was higher in experiment I than in experiment II. The reason for this was not clear, but in neither case was shown any difference between cultures with or without adherent cells.

Table 2. In vitro transformation of mouse lymphocytes in the presence or absence of adherent cells.

| <sup>14</sup> C-thymidine incorporation (c.p.m.) |                                |                |                 |                  |             |
|--|--------------------------------|----------------|-----------------|------------------|-------------|
|  | Cells<br>cultured<br>in medium | Serum-free     |                 | Serum-containing |             |
|  |                                | I <sup>+</sup> | II <sup>+</sup> | I                | II          |
| Lymph node cell<br>suspensions                   |                                |                |                 |                  |             |
| +ConA*   |                                | 8138 ± 1495    | 5776 ± 948      | 14214 ± 334      | 16738 ± 286 |
| containing<br>adherent<br>cells                  |                                |                |                 |                  |             |
| -ConA  |                                | 988 ± 10       | 223 ± 16        | 1060 ± 10        | 596 ± 8     |
| +ConA*   |                                | 7946 ± 2320    | 5565 ± 1047     | 14266 ± 984      | 16065 ± 817 |
| not<br>containing<br>adherent<br>cells           |                                |                |                 |                  |             |
| -ConA  |                                | 1076 ± 45      | 216 ± 11        | 1019 ± 15        | 606 ± 18    |

\* p > 0.1 in all cases. Mean counts ± S.D., + ConA, n = 7; -ConA, n = 4.

+ Two experiments were performed I & II.



## 2.5 DISCUSSION

Measurement of incorporation of radiolabelled precursors into DNA by mitogen stimulated lymphocytes has been used extensively to follow proliferation in vitro. The method measures an increased amount of biosynthetic activity following stimulation and therefore reflects directly the increased numbers of transformed cells. Proliferation also depends on the activity of macrophages; IL-1 production by macrophages and also antigen presentation during antigenic stimulation, play a crucial role in the proliferation of lymphocytes (Larsson et al., 1982; Rock & Benacerraf, 1983; 1984; Reske-kunz et al., 1984). However, as seen in the present investigation, macrophages did not incorporate labelled thymidine (Table 2), indicating that the accumulation of  $^{14}\text{C}$ -thymidine by stimulated mouse lymph node cells was the result of lymphocyte activity alone and not due to activity of these adherent cells.

In this study, it was found that maximum incorporation of  $^{14}\text{C}$ -thymidine in response to ConA by mouse lymph node cells in serum-free or serum-containing media occurred in 46-71 h cultures with a peak at 56 h (Fig. 2). In many cases lymphocytes have been cultured for a longer time before the degree of proliferation was assessed by measuring the incorporation of labelled thymidine into newly synthesized DNA (Joynson et al., 1972; Kulapongs et al., 1974; Phillips & Azari, 1974; Fletcher et al., 1975; Gross et al., 1975; Sawitsky et al., 1976; Brock, 1981; Van Heerden et al., 1981; Krantman et al., 1982; Novogrodsky et al., 1982; Soyono et al.,

1982; Kuvibidila et al., 1983a; Bonnet et al., 1984; Lipkowitz et al., 1984; Payan et al., 1984). However, most of these workers used peripheral human blood lymphocytes (Joynson et al., 1972; Kulapongs et al., 1974; Phillips & Azari, 1974; Fletcher et al., 1975; Gross et al., 1975; Van Heerden et al., 1981; Krantman et al., 1982; Novogrodsky et al., 1982; Payan et al., 1984), while mouse lymph node cells were used in the present investigation. Others have used lymphocytes from the spleens of rats (Soyano et al., 1982) or mice (Kuvibidila et al., 1983a,b). Brock (1981) used mouse lymph node cells, but test-tube cultures were used rather than the microculture method employed in the present investigation. In general, there is heterogeneity in the proliferative response of lymphocytes in vitro depending on the species, type and concentration of the mitogen (or antigen), and age of the animal from which the cells are isolated (Ling & Kay, 1975). Despite this heterogeneity it has been shown that the first period of DNA synthesis by stimulated lymphocytes occurs 48-72 h after addition of the mitogen (Sören, 1973; Ling & Kay, 1975), although a small proportion of cells may be early (24 h) or late (above 100 h) responders (Sören, 1973). Furthermore, Sasaki & Norman (1966) showed that 48-70 h cultures of lymphocytes consist of cells which are in the first cycle of division and that after 72 h stimulated cells are in the second or third metaphase. However, despite the fact that 72 h cultures have been more often used, in the present investigation, the cells were cultured for 55-69 h since maximum

proliferation of mouse lymph node lymphocytes occurred at this time (Fig. 2a ). In the absence of ConA the proliferative response in both media was very much lower than when it was present. However, there was still some incorporation of  $^{14}\text{C}$ -thymidine by the cells (Fig. 2b). This might have been due to repairing of DNA by some cells, or the result of non-specific stimulation of some lymphocytes. A few mesenteric lymph node cells might also have been antigenically stimulated prior to culture. The incorporation of labelled thymidine by unstimulated cultures was greater when the cells were cultured in medium containing FCS (Fig. 2b). Foetal calf serum has also been found to produce a considerable "background" stimulation of human lymphocytes, which in 7-day cell cultures, affected from 5-48% of the lymphocytes (Joynson & Russell, 1965). However, the "background" stimulation observed in both media was always very much lower than the response of the mitogen-containing samples. Serum-free medium, therefore, has an advantage over serum-containing medium in always giving low "background".

The incorporation of  $^{14}\text{C}$ -thymidine by lymph node cells cultured with ConA depended on the concentration of ConA used (Figs. 3,4). It is well established that the degree of lymphocyte proliferation in vitro depends on the strength of the stimulus received which in turn will depend on the number of molecules of mitogen bound to the cell surface receptors (Stobo et al., 1972; Ling & Kay, 1975). At concentrations of ConA above the optimum, however, the effect of the mitogen on the cell proliferation and

differentiation is inhibitory rather than stimulatory as indicated by the decreased incorporation of  $^{14}\text{C}$ -thymidine (Figs. 3,4). Concanavalin A is an agglutinin (Ling & Kay, 1975), so that at higher concentrations it may cause some agglutination of lymphocytes, and hence decreased responses. In addition, the binding of the mitogenic lectins has a distinctive effect on the mobility and distribution of all receptors on the lymphocyte cell-membrane (Yahara & Edelman, 1972; Cunningham et al., 1976). Depending on the conditions of cell-cultures, ConA has two antagonistic effects on the mobility of the cell receptors, and at high concentrations, it causes inhibition of cap formation by both its own receptors and other receptors, thus affecting receptor-cytoplasm interaction, and therefore mitogenesis (Yahara & Edelman, 1972; Wang et al., 1976). This typical unimodal dose-response curve seen in the mitogenic stimulation of lymphocytes has been also observed by other investigators (Ling & Kay, 1975; Cunningham et al., 1976; Stobo, 1977) and indicates the need to accurately determine the dose of ConA for optimum response. It was found, in the present investigation, that higher concentrations of ConA were needed to induce optimum transformation in serum-containing medium than when serum-free conditions were used (Figs. 3,4). This was probably because some ConA binds to serum proteins in the medium. It has been shown that mitogens such as PHA and ConA can react with  $\alpha_2$ -macroglobulin,  $\beta$ -lipoprotein and IgM and to a lesser extent with IgA (Morse, 1968; Powell & Leon, 1970; Ling &

Kay, 1975). Binding, therefore, of the mitogen to these molecules will cause a reduction in the total number of mitogen molecules available for lymphocyte activation. Since lymphocyte proliferation initially depends on the strength of mitogenic stimulus, higher concentrations of mitogen will, therefore, be needed to induce a proliferative response when serum proteins are present. In earlier experiments it was found that optimal transformation was obtained using 4  $\mu\text{g/ml}$  and 1  $\mu\text{g/ml}$  in serum-containing and serum-free media, respectively (Fig. 3). However, variation in the proliferative response of lymphocytes cultured in both media was observed when a second batch of ConA was needed later on (Fig. 4), and thereafter optimal transformation required concentrations of 2  $\mu\text{g/ml}$  and 0.5  $\mu\text{g/ml}$ , respectively.

The degree of proliferation of mouse lymph node cells cultured with ConA in serum-free conditions also depended on the saturation with iron of the transferrin present in the medium. Maximum proliferation was observed at a saturation of transferrin with iron of 70% (Fig. 5). Since, in vivo, saturation of mouse transferrin with iron is 65-80% (Puschmann & Ganzoni, 1977; Kuvibidila et al., 1983a; also Chapter 3), the above result may indicate that the saturation with iron of the transferrin added to the medium required to induce optimum proliferation of mouse lymphocytes in vitro may correspond to normal in vivo transferrin saturation. At lower saturations of transferrin, the proliferative response of lymphocytes was progressively reduced (Fig. 5). Brock (1981) also showed that the degree

of proliferation of mouse lymph node cells cultured in serum-free conditions depended on the saturation of transferrin with iron, and that maximum proliferation was observed at 30-70% saturation of transferrin with iron. However, he found no significant difference in the lymphocyte response when 30 or 50 per cent iron-saturated transferrin was used which is different to the results observed in the present investigation. This might have been due to the different experimental conditions employed. However, 30%-iron-saturated transferrin was subsequently used as a positive control for the reason that in a lymph node the saturation of transferrin with iron may be lower than that in the serum especially when a local response causes a rapid uptake of transferrin-bound iron, resulting in a local reduction of the saturation of transferrin.

The initial step in the uptake of iron involves the binding of the transferrin to specific cell membrane receptors and in rat hepatocytes (Young & Aisen, 1981) and in rabbit reticulocytes (Young et al., 1984b) the strength of interaction of transferrin with the receptor decreases from diferric to monoferric to apotransferrin. Furthermore, in human reticulocytes iron from diferric transferrin is more rapidly taken up by the cells than iron from monoferric transferrin (Huebers et al., 1981b; 1983). In consequence the degree of transformation of lymphocytes in medium containing partially saturated transferrin will most likely depend on the proportions of monoferric, diferric and apotransferrin molecules, hence the increased proliferation associated with increased percentage saturation of transferrin with

iron observed in the present investigation. From the data of Williams et al. (1978) it can be calculated that for ovotransferrin to which iron as Fe(NTA) in 0.1M - NaHCO<sub>3</sub> is added to saturate the transferrin by 70%, 50% of the molecules are expected to be in the diferric form and 40% and 10% would be monoferric and apotransferrin, respectively. However, at lower saturations of transferrin with iron the number of diferric molecules decrease and the number of monoferric and apotransferrin molecules will increase accordingly. Thus, the results suggest that the amount of iron bound to transferrin may be important in determining the degree of proliferation. However, at 100% saturation of transferrin with iron, the degree of transformation of mouse lymphocytes was lower than when the cells were cultured in medium containing 50 or 70 per cent iron-saturated transferrin (Fig. 5). Brock (1981) also reported similar results. It seems, therefore, that with mouse lymph node cells, the higher the saturation of transferrin with iron the better the in vitro proliferation until iron-saturation of transferrin reaches a level which corresponds to the in vivo situation. Above this level, the effect is reversed and transformation is reduced. Several explanations are possible. It has been suggested that transferrin-bound zinc is also important for lymphocyte proliferation (Phillips, 1978) and since iron binds to transferrin with higher affinity than zinc (Fraenkel-Conrat & Feeney, 1950; Warner and Weber, 1953; Tan & Woodworth, 1969) saturation with iron will prevent the binding of zinc. However, Chesters and Will

(1981) found that albumin was the major transport protein for zinc in the plasma and not transferrin, and they showed that zinc-binding to transferrin was quantitatively insignificant. Another possible explanation arises from the observation that lymphocytes, unlike many other types of cells, do not synthesize ferritin in response to increased levels of iron (Summers et al., 1975; Summers and Jacobs, 1976; Lema & Sarcone, 1981). At high transferrin saturation with iron, uptake may, therefore, proceed too rapidly to allow adequate intracellular processing, with possible toxic effects and a consequent reduction of transformation.

In the present investigation, it was also found that the degree of proliferation of mouse lymph node lymphocytes cultured in serum-free medium containing either mouse or human transferrins of the same iron-saturation was not statistically different (Table 1). This indicates that human transferrin is as good as mouse transferrin in promoting proliferation of mouse lymphocytes and that the transferrin receptor in mouse lymph node cells could interact equally well with either human or mouse transferrin. Human transferrin can, therefore, be substituted for mouse for in vitro lymphocyte cultures in serum-free conditions. Brock (1981) also showed that human transferrin induced similar proliferative response as mouse transferrin when lymph node cells were cultured in serum-free conditions.



CHAPTER 3

THE EFFECT OF IRON DEFICIENCY ON THE

IN VITRO TRANSFORMATION OF MOUSE

LYMPH NODE LYMPHOCYTES

### 3.1 INTRODUCTION

Iron deficiency has often been associated with decreased cell-mediated immune responses. Clinical studies have demonstrated impaired delayed type hypersensitivity to PHA (Talwalkar et al., 1982) and PPD of Mycobacterium tuberculosis, Candida antigens (Joynson et al., 1972), diphtheria toxoid and streptokinase-streptodornase (Macdougall et al., 1975) and to Trichophyton antigens (Chandra & Saraya, 1975) in humans with iron deficiency anaemia. Furthermore, impaired in vitro blastogenic activity of peripheral blood lymphocytes to PPD and Candida antigens (Joynson et al., 1972), pokeweed mitogen (PWM; Sawitsky et al., 1976), PHA (Bhaskaram & Reddy, 1975; Chandra and Saraya, 1975; Fletcher et al., 1975; Macdougall et al., 1975; Sawitsky et al., 1976; Van Heerden et al., 1981) or ConA (Van Heerden et al., 1981) have been reported in iron deficient anaemic adults and children. Children with latent iron deficiency also showed reduced skin test responses to diphtheria toxoid and streptokinase-streptodornase, and reduced blastogenic responses to PHA (Macdougall et al., 1975). Lymphocytic function as assessed by leucocyte migration inhibition test was also impaired in iron deficient anaemic adults (Talwalkar et al., 1982). The proportion of circulating T-lymphocytes has also been found to be reduced in iron-deficient anaemic adults (Bhaskaram & Reddy, 1975; Chandra & Saraya, 1975; Fletcher et al., 1975) and in children with latent iron deficiency with or without anaemia

(Srikantia et al., 1976). Iron therapy increased the proportion of T-lymphocytes (Bhaskaram & Reddy, 1975; Fletcher et al., 1975) and lymphocyte proliferation in vitro (Fletcher et al., 1975; Macdougall et al., 1975; Sawitsky et al., 1976) back to normal values, although in one study no change in  $^3\text{H}$ -thymidine uptake was observed after iron therapy (Bhaskaram & Reddy, 1975). In addition, iron repletion in vivo caused an increase in delayed hypersensitivity to PHA and also improved secretion of migration inhibition factor by lymphocytes (Talwalkar et al., 1982). Animal studies have also shown that cell mediated immune responses may be affected in iron deficiency. Delayed cutaneous hypersensitivity reactions of iron-deficient anaemic mice to dinitrofluorobenzene, measured as the rate of incorporation of iodinated DNA-precursors in the sensitised area was markedly decreased when compared to control or paired groups (Kuvibidila et al., 1981). Injection of iron prior to the recall dose restored the rate of DNA-synthesis in the anaemic mice but had no effect on either the control or paired groups. Furthermore, the response of splenic lymphocytes from iron-deficient mice to PHA, ConA and lipopolysaccharide (LPS) was significantly impaired when compared with control mice (Kuvibidila et al., 1983a, b) and there was also a small but significant decrease in the number of peripheral blood lymphocytes (Kuvibidila et al., 1983a). Addition of mouse transferrin (unspecified saturation) or ferric chloride to the culture medium partially restored

the response of the cells to PHA but not to ConA and LPS, while haemin restored responses to both PHA and ConA (Kuvibidila et al., 1983b). However, no explanation was offered by the authors of the different responses of the lymphocytes under the different conditions employed. Furthermore, the in vivo repletion of iron levels of the iron-deficient group resulted in the in vitro restoration of the blastogenic response of lymphocytes to PHA, while the response to ConA and LPS was improved but not fully restored (Kuvibidila et al., 1983a). In contrast to the above findings Soyano et al. (1982) found a marked increase in the proliferative responses to PHA, ConA and PWM of splenic lymphocytes from iron-deficient rats whereas the response of thymic lymphocytes was very much impaired. It was suggested that this difference could have been the result of an alteration of the cell populations in the various lymphoid compartments.

It appears therefore, that iron deficiency may affect cell-mediated immunity in vivo or in vitro in both man and experimental animals. However, there have also been reports of normal cell-mediated immune responses and blastogenic activity of lymphocytes in iron deficiency. Delayed type hypersensitivity reactions to dinitrochlorobenzene in iron-deficient patients were found to be the same as in the control groups (Gross et al., 1975). Furthermore, the proliferative responses of peripheral blood lymphocytes to PHA or ConA in iron-deficient patients (Gross et al., 1975; Srikanthia et al., 1976; Van Heerden

et al., 1981) and in children with iron deficiency anaemia, before and after iron therapy (Kulapongs et al., 1974) did not significantly differ from that of the control groups. Krantman et al. (1982) and Gupta et al. (1982) also found that the response to PHA of lymphocytes from iron-deficient anaemic children were within the normal range. However, there was an increased responsiveness of the cells after iron therapy (Krantman et al., 1982). The proportion of T-lymphocytes has also been reported to be within normal range in iron deficiency alone (Van Heerden et al., 1981) or with anaemia (Van Heerden et al., 1981; Krantman et al., 1982), which conflicts with reports cited earlier indicating a decreased proportion of T-cells. Furthermore, Srikanthia et al. (1976) found that children with anaemia and normal transferrin saturation had an unexpectedly lower T-cell count than children who had evidence of both anaemia and latent iron deficiency.

The precise cellular or molecular defects in lymphocytes that may be induced by or associated with iron deficiency remain unclear. One of the reasons for this may be the lack of control of other factors which may affect the immune status. The presence of infections (Mendes et al., 1971; Mendes & Raphael, 1971) or the use of anti-inflammatory drugs (Tormey et al., 1967) or immunosuppressive agents (Baxter & Harris, 1975; White et al., 1979) have been known to depress cell-mediated immunity. Deficiencies of other nutritional factors such as zinc (Cunningham-Rundles et al., 1979; Gross et al., 1979; Pekarek et al.,

1979; Beisel, 1982) or copper (Beisel, 1982) or deficiency of vitamin A (Krishnan et al., 1974; Bhaskaram & Reddy, 1975; Levis & Emden, 1976) and vitamin B<sub>12</sub> (MacCuish et al., 1974; Das & Herbert, 1978) have also been shown to depress cell-mediated immune responses. In some reports on the in vitro or in vivo cell-mediated immune responses in iron deficiency in humans, existence of infections, malignancies, usage of immunosuppressive agents by the patients or signs of protein or vitamin deficiencies were not excluded (Bhaskaram & Reddy, 1975; Chandra & Saraya, 1975). In other studies, although clinical signs of infection and chronic illness (MacDougall et al., 1975; Srikanthia et al., 1976; Krantman et al., 1982) were excluded, vitamin or other biochemical deficiencies were not looked for and could affect interpretation of their findings. Since infections and other nutritional factors which affect cell-mediated immunity are difficult to control in human studies, it may be preferable to study the in vitro or in vivo cell-mediated immune responses in an experimental animal model in which infections and presence of deficiencies other than iron are better controlled, and age and sex matching more easily achieved. Although animal studies have been carried out before (Soyano et al., 1982; Kuvibidila et al., 1983a, b) these studies have not provided an adequate explanation for the observed changes in lymphocyte transformation resulting from iron deficiency.

The biochemical basis of reduced cell-mediated immune responses and in vitro lymphocyte transformation which may be associated with iron deficiency has not been elucidated. A factor which may be significant is the reduction in levels of iron available to lymphocytes for mitosis and DNA synthesis. Hershko et al. (1970) showed that DNA synthesis by bone marrow cells is reduced in iron deficiency, and it is known that iron is required for the activity of the enzyme ribonucleotide reductase which is involved in the reduction of ribonucleoside diphosphates to deoxyribonucleotides (See 1.1.6). It is possible that the reduced lymphocyte proliferation may result from defective DNA synthesis due to inadequate levels or activity of ribonucleotide reductase. It is therefore critical in elucidating the effect of iron deficiency on lymphocyte responses in vitro to use an experimental system which allows limitation of iron supply in the culture of lymphocytes. The controversial nature of the data on the correlation of iron deficiency and lymphocyte proliferation in vitro reported in the literature may therefore have arisen due to the fact that while most investigators used normal serum or foetal calf serum in their lymphocyte cultures, others used autologous serum. The iron content of these sera would undoubtedly vary. However, it is now possible to study the in vitro mitogenic response of lymphocytes using serum-free conditions (Dillner-Centerlind et al., 1979; Brock, 1981) which allow iron levels to be accurately controlled.

This study was carried out in an attempt to define more closely the effect of iron deficiency, and in particular the role of circulating (i.e. transferrin-bound) iron on lymphocyte transformation. To ensure the absence of environmental or other nutritional factors which might influence results, an experimental animal model consisting of mice fed on defined diets of known iron content was used. Furthermore, iron levels in the culture media were controlled using both serum-free and serum-containing media, in order to relate lymphocyte transformation to the availability of transferrin-bound iron.



### 3.2 MATERIALS

3.2.1. Mice. Unless otherwise stated, female 7-8 weeks old mice were used throughout the experiments described in this chapter.

3.2.2. Diet. Information for the formulation and preparation of the semisynthetic diet was kindly provided by Dr C.F. Mills, Department of Inorganic Biochemistry, Rowett Research Institute, Aberdeen. Table 3 lists the ingredients of the complete diet. Table 4 lists the resulting concentrations of the essential elements. To prepare the diet, the casein and methionine were mixed with two-thirds of the sucrose. The remaining sucrose was put aside for pre-mixing the other supplements. To the basic diet, finely ground mineral supplements (elements 14-18, Table 4) were added. The metasilicate was powdered in a coffee-mill in a fume-hood and mixed with sucrose before being added to the diet. Major trace elements (Cu, Zn, Mn and when necessary, Fe) were powdered and mixed with another portion of sucrose before addition to the diet. The minor trace element mix (Table 3) which was made in a sucrose carrier (4.97 g sucrose) was prepared in bulk and stored at 4°C in a tightly stoppered polythene jar. The sucrose/minor trace element mix was ground with more sucrose before being added to the diet. The water soluble vitamins were first dispersed in sucrose (0.5 g) and inositol, then ground to a fine powder in a mortar and mixed with more sucrose before

Table 3: Complete diet (1 kg).

| Ingredient  | Weight of ingredient |
|---|----------------------|
| Casein (60 mesh)  | 200.00 g             |
| Methionine (Sigma)  | 5.80 g               |
| Commercial sucrose  | 648.00 g             |
| Inositol (Sigma)  | 0.40 g               |
| Arachis oil (Pharmacy, Gartnavel<br>General Hospital,<br>Glasgow) | 100.00 g             |
| CaCO <sub>3</sub>   | 15.00 g              |
| Na <sub>2</sub> H <sub>3</sub> PO <sub>4</sub>                    | 6.60 g               |
| KH <sub>2</sub> PO <sub>4</sub>                                   | 15.70 g              |
| KCl   | 1.10 g               |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O                              | 5.10 g               |
| (Na <sub>2</sub> SiO <sub>3</sub> ·5H <sub>2</sub> O              | 0.76 g               |
| (NaIO <sub>3</sub> ·H <sub>2</sub> O                              | 1.70 mg              |
| (NaF  | 5.50 mg              |
| (V <sub>2</sub> O <sub>5</sub>                                    | 0.27 mg              |
| (NiSO <sub>4</sub>  | 2.60 mg              |
| (SnCl <sub>4</sub>  | 3.20 mg              |
| (K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>                    | 14.00 mg             |
| (Na <sub>2</sub> SeO <sub>3</sub>                                 | 0.23 mg              |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O                              | 19.70 mg             |
| FeSO <sub>4</sub> ·7H <sub>2</sub> O*                             | 249.00 mg            |
| MnSO <sub>4</sub> ·4H <sub>2</sub> O                              | 203.00 mg            |
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O                              | 176.00 mg            |
| Thiamine <sup>2</sup> (Sigma)                                     | 10.00 mg             |
| Pyridoxine (Sigma)  | 10.00 mg             |
| Riboflavine (Sigma)   | 10.00 mg             |
| p-aminobenzoic acid (Sigma)                                       | 10.00 mg             |
| Nicotinic acid (Sigma)  | 30.00 mg             |
| Ca-pantothenate (Sigma)   | 20.00 mg             |
| Folic acid (Sigma)  | 5.00 mg              |
| Biotin (Sigma)  | 5.00 mg              |
| Tocopherol acetate (Vit.E; Sigma)                                 | 0.20 g               |
| Retinylacetate (Vit. A; Sigma)                                    | 0.80 mg              |
| Calciferol (Vit. D; Sigma)  | 0.27 mg              |
| Choline chloride (BDH Chemicals Ltd.)                             | 1.00 g               |
| Vitamine B <sub>12</sub> (Sigma)                                  | 25.00 µg             |
| Menadione (Vit. K <sub>3</sub> ; Sigma)                           | 5.00 mg              |

Minor  
trace  
element  
mix

\* Added as appropriate.

Table 4: Essential elements and their dietary concentrations.

| No. | Element | Dietary<br>Concentration<br>(mg/kg) |
|-----|---------|-------------------------------------|
| 1   | Cu      | 5.0                                 |
| 2   | Fe*     | 50.0                                |
| 3   | Mn      | 50.0                                |
| 4   | Zn      | 40.0                                |
| 5   | Si      | 100.0                               |
| 6   | I       | 1.0                                 |
| 7   | F       | 2.5                                 |
| 8   | V       | 0.2                                 |
| 9   | Ni      | 1.0                                 |
| 10  | Sn      | 2.0                                 |
| 11  | Se      | 0.1                                 |
| 12  | Cr      | 5.0                                 |
|     |         | (g/kg)                              |
| 13  | Ca      | 6.0                                 |
| 14  | K       | 5.1                                 |
| 15  | Mg      | 0.5                                 |
| 16  | Na      | 2.3                                 |
| 17  | P       | 5.0                                 |
| 18  | Cl      | 0.5                                 |

\* Added as appropriate.

addition to the diet. Choline chloride was mixed with vitamin B<sub>12</sub> and the remainder of the sucrose before being added to the diet. The water insoluble vitamins consisting of vitamins A, D, K<sub>3</sub> and E were dissolved in chloroform (1 ml) and mixed with the arachis oil before addition to the diet. The complete diet was thoroughly mixed and stored at -4°C in portions of 500 g. Enough diet was prepared to allow 5 g diet per mouse per day. The diet was used within 6 weeks. Two diets were prepared, one containing FeSO<sub>4</sub> and the other without iron. The iron-content of the iron-deficient diet was kindly estimated by Dr D. Halls using carbon furnace atomic absorption spectroscopy and was found to be 820 µg/kg. The diet supplemented with FeSO<sub>4</sub> contained an iron level of 50 mg/kg.

All glassware used for the preparation of the iron-deficient diet were rendered iron-free prior to use (see 2.3.1.2).

### 3.2.3. Reagents for estimating the iron status of mice

3.2.3.1. Acid digest solution. This solution, used for the extraction of non-haem iron from the mouse liver samples, was prepared by dissolving 10 g of trichloroacetic acid (TCA; Analytical reagents grade, M & B Ltd., Dagenham, Essex, England) in d-DW (70 ml) and adding 29.7 ml of concentrated HCl (Analar grade, BDH).

3.2.3.2. Protein precipitant solution. This contained 10% (w/v) TCA, 3% (v/v) thioglycolic acid (BDH) and 2M HCl. It was stored in the dark in an iron-free glass universal

and used diluted 1:3 in d-DW.

3.2.3.3. Ferrozine chromogen. This was prepared by dissolving 105 mg of ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-thiazine, BDH) in a drop of d-DW and then adding 100 ml of saturated sodium acetate (Analar grade, BDH) made in d-DW.

3.2.4. Reagents for estimating haemoglobin levels

A kit was supplied by Sigma (Kit No. 525).

3.2.4.1. Reagents supplied in the kit

3.2.4.1.1. Drabkin's reagent (Stock No. 525-2). This was a dry mixture consisting of sodium bicarbonate (100 parts), potassium ferricyanide (20 parts) and potassium cyanide (5 parts).

3.2.4.1.2. Brij-35 solution (Stock No. 430 Ag-6). This solution contained Brij-35, 30 g/100 ml.

3.2.4.1.3. Haemoglobin standard (Stock No. 525-18). This contained lyophilized human haemoglobin equivalent to 18 g ( $\pm$  1%) haemoglobin per 100 ml whole blood when reconstituted with Drabkin's solution.

3.2.4.2. Materials prepared using reagents supplied in the kit

3.2.4.2.1. Drabkin's solution. This was obtained by reconstituting Drabkin's reagents with 1000 ml water. To this, 0.5 ml of 30% Brij-35 solution was added and mixed well. The solution was filtered and stored in the dark at room temperature.

3.2.4.2.2. Cyanmethaemoglobin solution. This solution was obtained by reconstituting the haemoglobin standard with 50 ml Drabkin's solution. The solution was mixed well and left to stand for 30 min at room temperature before use.

3.2.5. Reagents for estimating protein synthesis

Minimum essential complete medium. Minimum essential medium without leucine (Flow) was supplemented with penicillin, streptomycin, L-glutamine and 2-Me as described for the RPMI-1640 culture medium (see 2.2.2.7). To this were added 10  $\mu$ Ci/ml of tritiated leucine (specific activity 160 Ci/mMol; Amersham) and 40  $\mu$ M (final concentration) cold leucine (BDH). Apotransferrin or 20%-iron-saturated transferrin (50  $\mu$ g/ml) or foetal calf serum (2%) (Gibco) were added as appropriate.

3.2.6. Reagents for estimating numbers of T-cells and T-cell subsets.

3.2.6.1. Formol calcium solution. This solution (pH 6.7) contained 10% formaldehyde and 1%  $\text{CaCl}_2$  in DW.

3.2.6.2. Phosphate buffer (0.06M). This was prepared by mixing 1.5 ml of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (11.88 g/l) with 98.5 ml  $\text{KH}_2\text{PO}_4$  (9.08 g/l).

3.2.6.3. Hexazotised pararosaniline. This solution was formed by mixing equal volumes of solution A and solution B as below:

Solution A - 4%  $\text{NaNO}_2$  in DW, freshly prepared.

Solution B - 1g pararosaniline (Sigma) was dissolved in 20 ml warm DW and 5 ml of concentrated HCl were added. This solution was allowed to cool and was filtered before use.

3.2.6.4.  $\alpha$ -naphthyl acetate esterase (ANAE) reagent. This consisted of 40 ml of 0.06M phosphate buffer (pH 5.0), 2.4 ml of hexazotised pararosaniline solution and 10 mg of  $\alpha$ -naphthyl acetate (Sigma) which was dissolved in 0.4 ml acetone prior to addition to the buffer-pararosaniline mixture. The pH of the complete reagent was adjusted to 5.8 with 2M NaOH. The solution was filtered before use.

### 3.3 METHODS

#### 3.3.1. Induction of iron deficiency

Twenty mice were assigned to four groups and housed individually in grid-bottomed cages. The mice were fed for six weeks on either laboratory diet (ND) (Rat and Mouse No.1 Maintenance Expanded Diet; Special Diet Services, Witham, Essex, England), iron-supplemented semisynthetic diet (SD), low-iron semisynthetic diet (DD) or pairfed with the iron-supplemented semisynthetic diet (PF). All animals were given diet ad libitum except the PF group which received an amount equal to the average amount consumed by the DD group the previous day. This group was included in order to allow for the possible effects of protein-calorie malnutrition. Tap water was given to ND, SD and PF groups while the DD group received d-DW. Glass feeding dishes used for the DD group were acid-washed before use. The mice were weighed weekly and the diet was replaced daily.

#### 3.3.2. Iron repletion

For iron repletion studies, the mice were assigned to three groups (1 to 3), and fed either on iron-supplemented diet (group 1) or low-iron diet (groups 2 and 3) for six weeks, after which group 3 was put on iron-supplemented diet. The mice were fed on corresponding diets for a further four weeks. Mice receiving low-iron diet at any time were given d-DW, while tap water was used for mice fed on iron-supplemented diet.



### 3.3.3. Sacrifice of mice

At the end of the feeding period, a blood sample was drawn by heart puncture and the mice were then immediately killed by cervical dislocation. Lymph nodes (see 2.3.2) and livers were removed. The blood samples were used, after clotting, for determination of serum iron and total iron binding capacity (TIBC), while unclotted samples were used for estimating haemoglobin levels. The livers were used for determining storage iron and the lymph nodes to prepare the lymphocyte suspensions (see 2.3.2). One mouse per group was killed per day over a period of the required number of consecutive days.

### 3.3.4. Culture conditions and mitogen stimulation

Cell suspensions were prepared as described in Section 2.3.2. The cells were cultured in either serum-containing culture medium or serum-free medium containing HSA and human transferrin (see 2.2.2.7) either as apotransferrin or 20%-iron-saturated transferrin (see 2.2.2.6). However, taking into account the iron content of the serum-free medium which was 12.5 ng/ml (see 2.3.1.1), the final saturations of transferrin with iron were 10% and 30% respectively. The cells were cultured as described in Section 2.3.2 with ConA at a concentration of either 4 or 1 µg/ml for medium containing FCS or transferrin, respectively, and for a period of 51-52 h before pulsing with labelled thymidine for 4 h. Assaying of proliferative responses was carried out as described elsewhere (see 2.3.2).

### 3.3.5. Determination of serum iron

Serum was prepared by centrifugation (twice) of the clotted blood samples in a MSE Centaur 2 centrifuge at 3000 r.p.m. for 10 min. The serum iron was estimated by a method based on that of Brittenham (1979). The method which was used because it is designed for small volumes of serum, is a colorimetric one and involves precipitation of serum proteins, dissociation of iron from transferrin and formation of a complex of reduced iron with a chromogen. Mouse serum (100  $\mu$ l) and protein precipitant (see 3.2.3.2) (300  $\mu$ l) were pipetted into 1.5 ml Eppendorf microtubes (Anderman & Co.). The tubes were capped, thoroughly vortexed and, after standing for 5 min, centrifuged for 10 min in a microchaematocrit centrifuge (Hawksley, England). Supernatants of 320  $\mu$ l were removed into clean microtubes and 80  $\mu$ l of ferrozine chromogen solution (see 3.2.3.3) were added. The tubes were vortexed and after standing for 5 min the absorbance at 562 nm was determined using microcuvettes in an ultrospec 4050 spectrophotometer (LKB, Cambridge, England). The microcuvettes were rinsed in diluted protein precipitant before use. Iron standard solutions (70 - 280  $\mu$ g/100 ml) were prepared using  $\text{Fe(NTA)}$  solution and d-DW, and also d-DW was used instead of serum for the reagent blank. The straight line was fitted to the absorbance versus iron levels plot by the method of least squares and the serum values were calculated from the regression line. All assays were performed in duplicate.

### 3.3.6. Determination of total iron binding capacity

To a serum sample (300  $\mu$ l) in an iron-free polypropylene test tube (see 2.3.1.2) 300  $\mu$ l of 100  $\mu$ M Fe(NTA) solution was added. The mixture was thoroughly vortexed and after standing at room temperature for 15 min, the excess unbound iron was removed using hydrated  $\text{MgCO}_3$  (0.045 g; BDH) which was added with a previously calibrated scoop. The test tubes were stoppered and mixed on a rotating shaker (Hoechst) for 45 min. The samples were then centrifuged for 15 min at 2500 r.p.m. and thereafter the supernatants were treated as in serum iron assays (Section 3.3.5). Reagent blank and iron standard solutions were prepared as in the serum assays. Since insufficient serum could be obtained from individual mice for both serum and total iron binding capacity, TIBC values were obtained using pooled sera of mice of the same group.

### 3.3.7. Estimation of haemoglobin levels

Total haemoglobin levels were estimated colorimetrically by the cyanmethaemoglobin method using a kit (Section 3.2.4). The method involves oxidation of haemoglobin to methaemoglobin at alkaline pH and in the presence of potassium ferricyanide. Methaemoglobin when reacting with potassium cyanide forms cyanmethaemoglobin which gives maximum absorption at 540 nm. Cyanmethaemoglobin standards were prepared by diluting the cyanmethaemoglobin solution (Section 3.2.4.2.2.) with Drabkin's solution (Section 3.2.4.2.1) to get preparations containing 6, 12 and 18 g haemoglobin per 100 ml of whole

blood. The absorbance of the standard solutions were read at 540 nm against Drabkin's solution as a reagent blank in the Ultrospec 4050 spectrophotometer and a calibration curve constructed. For the test samples, 20  $\mu$ l of whole blood were mixed well with Drabkin's solution (5 ml) and, after standing at room temperature for 15 min, they were read against Drabkin's solution. Total haemoglobin concentrations of the test blood samples were determined directly from the calibration curve. Samples were assayed in duplicate. Due to the small volume of blood available, it was not possible to assay for both haemoglobin and serum iron/TIBC on samples from the same animal.

#### 3.3.8. Determination of liver iron

To extract non-haem liver iron, liver samples (0.2 g) were cut into small pieces and heated for 20 h at 65°C in 1 ml of acid digest solution (Section 3.2.3.1). After cooling to room temperature, 50  $\mu$ l of the extract was transferred to an Eppendorf microtube and d-DW (50  $\mu$ l) and diluted protein precipitant solution (300  $\mu$ l) were added. Thereafter, the serum iron procedure was followed. All samples were prepared in duplicate using two different samples of liver for each mouse. The standard solutions were prepared as described elsewhere (see 3.3.5).

#### 3.3.9. Determination of protein synthesis by transformed lymphocytes

Mouse lymph node cells were cultured for 55-56 h with ConA in serum-free media containing apotransferrin or 20%-

iron-saturated transferrin (see 2.3.2). The transformed cells were washed twice (1200 r.p.m., 45-50 sec) with warm (37°C) Hanks balanced salts solution (Gibco) containing either FCS (2%), or 10%- or 30%-iron-saturated transferrin (50 µg/ml), as appropriate. After washing, the cells were resuspended at a concentration of  $5 \times 10^6$  viable cells/ml in  $^3\text{H}$ -leucine-containing minimum essential complete medium (Section 3.2.5). Aliquots (100 µl) of the transformed cell suspensions were cultured in microtitre plates for 1 h, after which 100 µl of ice-cold PBS containing 1 mM L-leucine was added to each well and the plates were chilled at 0°C for 15 min. The cells were then harvested by suction through 2.5 cm diameter glass microfibre filters (Whatman). Each well was rinsed with PBS and the cells were washed twice with PBS. The DNA was extracted from the cells with 5% trichloroacetic acid. The filter mats were finally washed with methylated spirit and, after drying at 50°C for 1 h, the tritium activity was measured in the liquid scintillation counter. The number of replicates depended on the initial density of the transformed cells and varied between 2 and 12.

#### 3.3.10. α-naphthyl acetate esterase staining

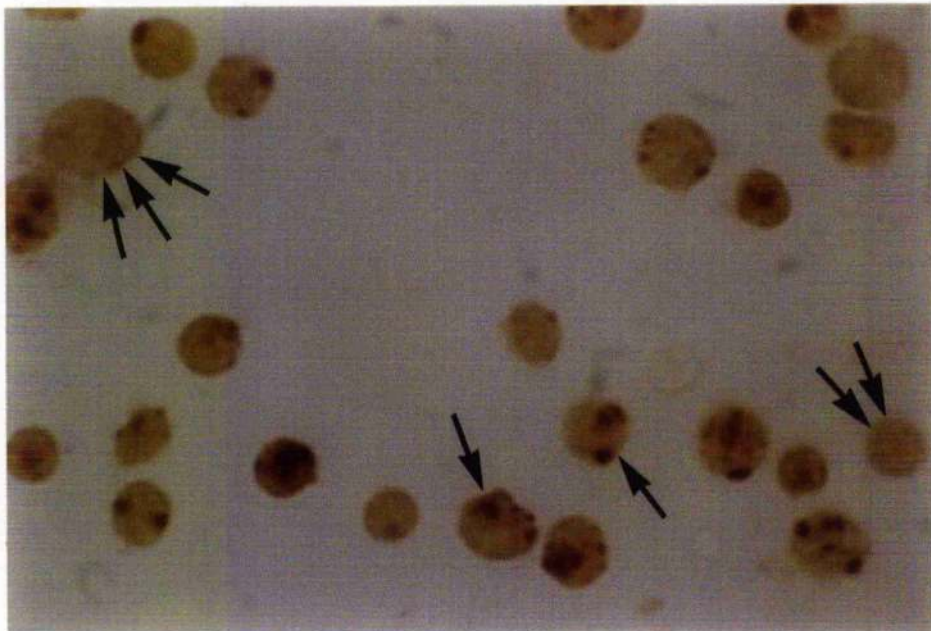
About  $6 \times 10^5$  lymph node cells were deposited on to clean glass slides in a Shandon cytocentrifuge at 1000 r.p.m. for 90 sec. The preparations were air-dried and fixed in formol calcium (pH 6.7) at 4°C for 10 min. The slides were then washed in running water for 20 min and finally stained with ANAE-reagent (see 3.2.6.4) for 90 min at 37°C. After

staining, the slides were washed in gently running water for 10 min and, after being dried, counterstained with 2% methyl green for 30 sec. The slides were then rinsed in tap water, dried, mounted with DPX and finally examined using a Leitz-Welzlar microscope (x 400). Using this stain, T-lymphocytes are characterised by small brown spots in their cytoplasm, monocytes and macrophages show a diffuse brown staining throughout the whole cell, and B-lymphocytes (and null cells) do not stain (Fig. 6). At least three fields chosen at random were examined and the percentage of T-lymphocytes was calculated.

#### 3.3.11. Immunofluorescent staining of T-cells

T-cells and T-cell subsets can be recognized with monoclonal antibodies to cell markers. In mice, monoclonal antibodies to Thy<sub>1,2</sub> cell antigens distinguish T-lymphocytes from B-lymphocytes and macrophages or monocytes, while  $\alpha$ -Ly<sub>1</sub> and  $\alpha$ -Ly<sub>2</sub>, probably recognize T-helper and T-suppressor cells, respectively. Fluorescein-labelled monoclonal antibodies to Thy<sub>1,2</sub>, Ly<sub>1</sub> and Ly<sub>2</sub> T-cell antigens were obtained from Becton-Dickinson (Sunnyvale, Ca., U.S.A.) and used diluted in PBS at 1:20, 1:20 and 1:4 respectively. Cytocentrifuge preparations of cells were obtained as described in Section 3.3.10. The slides were fixed in freshly made formalin (2.5% in DW) for 10 min and washed in PBS for the same time before staining with 15  $\mu$ l of the appropriate dilution of the monoclonal antibody for 30-40 min in a moist box in the dark at 4°C. After staining

Figure 6: A population of cells from mesenteric and peripheral lymph nodes of mice stained with  $\alpha$ -naphthyl acetate esterase stain.



Cells indicated by one arrow are T-lymphocytes, double arrows point to an ANAE-stain negative cell (B-lymphocyte or null cell) and triple arrows to a monocyte or macrophage. (x 900).

the slides were washed three times (10 min each time) in cold PBS containing 0.02% azide and then mounted in PBS-glycerol (4 parts PBS:1 part glycerol). More than 300 cells from different fields chosen at random were counted under phase contrast and U.V. using a Leitz Ortholux microscope. The percentage of positively-stained cells was calculated for each antibody and finally the number of cells positive for  $ly_1$  and  $ly_2$  surface antigens was calculated as a percentage of cells positive for  $thy_{1,2}$  antigen.



### 3.4 RESULTS

#### 3.4.1. Mouse weights

The weight gain at the end of the feeding period of all mice fed on different diets (apart from mice used for the iron repletion studies) was analysed using two-way analysis of variance (two-way ANOVA).

The mean weight gain of mice fed on laboratory diet was significantly lower than the mean weight gain of mice fed on the semisynthetic diet (Fig. 7, Table 5). This was probably the result of the different carbohydrate content of the laboratory diet in comparison with that of the semisynthetic diet (see 3.5). However, there was no significant difference in mean weight gain among the mice fed on any semisynthetic diet (Fig. 7, Table 6) indicating that the low-iron diet did not cause weight loss. There was also an experiment-to-experiment variation of statistical significance in weight gain in mice fed on different diets (Tables 5 and 6), which was not the result of the effect of the diet on the mice (Table 6). The small but significant effect seen in the interaction of diet and experiment (Table 5) was probably due to the difference observed between laboratory and semisynthetic diet (Fig. 7, Table 5).

The weight gain of mice which were used for iron repletion studies were analysed using Scheffé's method of multiple comparisons (see Appendix). Two analyses were performed of:

Figure 7: Weight gain observed in mice fed on different diets.  
(Results of eight experiments pooled).

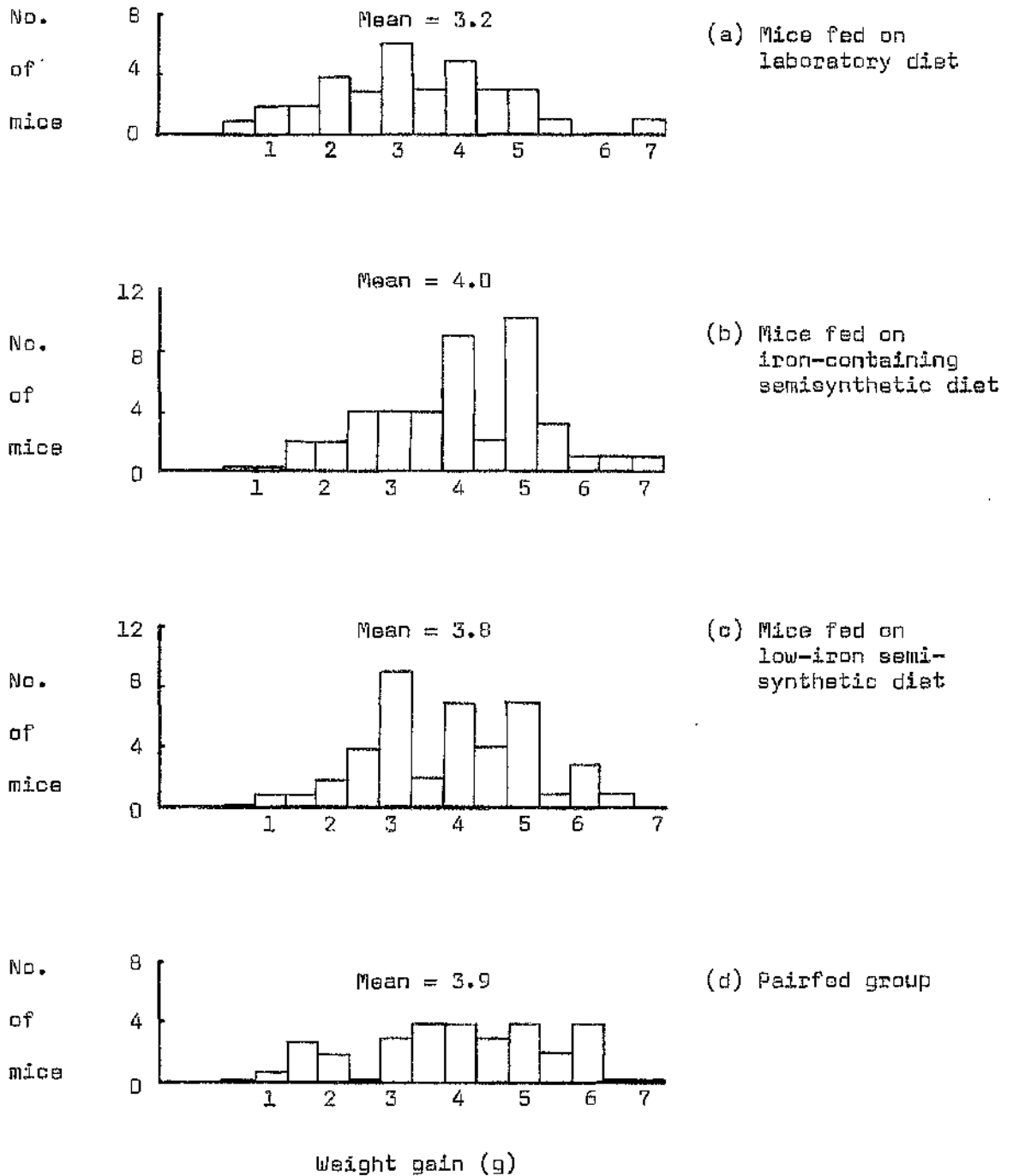


Table 5: Two-way analysis of variance of weight-gain of all mice fed on laboratory diet, iron-containing semisynthetic diet, low-iron diet and pairfed.

| Due to                             | F ratio | Significance |
|------------------------------------|---------|--------------|
| Day-to-day variation               | 11.141  | $p < 0.001$  |
| Diet                               | 3.376   | $p < 0.05$   |
| Interaction of diet and experiment | 1.734   | $p < 0.05$   |

Table 6: Two-way analysis of variance of weight-gain of mice fed on iron-containing diet, low-iron diet and paired.

| Due to                             | F ratio | Significance |
|------------------------------------|---------|--------------|
| Day-to-day variation               | 9.569   | $p < 0.001$  |
| Diet                               | 0.534   | ns           |
| Interaction of diet and experiment | 1.490   | ns           |

1. the weight gain during the first 6 weeks feeding period and
2. the weight gain during the period between 6th and 10th week.

Both analyses showed that there was no significant difference in weight gain between mice fed on different diets (Table 7).

3.4.2. Effect of iron deficiency on the transformation of mouse lymph node lymphocytes cultured in serum-free medium and in medium containing foetal calf serum

3.4.2.1. Transformation of lymphocytes in serum-free medium

3.4.2.1.1. Iron status

The serum and liver iron levels of the DD group were significantly lower compared with the other three groups of mice. TIBC values of the DD group were higher than in the other groups so that the percentage saturation of serum transferrin with iron in the DD group was very much reduced (Tables 8 and 9). These results indicated that iron deficiency was established in the DD group at the end of the six-week feeding period.

3.4.2.1.2. Mitogenic response of lymphocytes

The degree of transformation of lymphocytes in culture medium containing 10%-iron-saturated transferrin was significantly lower in all groups compared with the responses of the cells cultured in medium containing 30%-iron-saturated transferrin (Table 10). These results indicated that the iron content of the culture medium was an important factor controlling the degree of mitogenic response. However, although there was a lower mitogenic response of lymphocytes of the DD group in medium containing 10%-iron-saturated

Table 7: Analysis by Scheffé's method of multiple comparisons of the weight gain of mice used for iron-repletion studies.

| Groups compared* | Simultaneous 95% confidence limits |                            | Significant difference |  |
|------------------|------------------------------------|----------------------------|------------------------|--|
|                  | Week                               | 1 - 6      6 - 10          | 1 - 6      6 - 10      |  |
| 1 & 2            |                                    | 1.50,-2.51      1.90,-1.20 | ns      ns             |  |
| 1 & 3            |                                    | 2.10,-1.92      2.80,-0.35 | ns      ns             |  |
| 2 & 3            |                                    | 2.75,-1.30      2.41,-0.62 | ns      ns             |  |

\* 1: mice fed on iron-containing semisynthetic diet for 10 weeks;

2: mice fed on low-iron diet for 6 weeks and then on iron-containing diet for 4 weeks;

3: mice fed on low-iron diet for 10 weeks.

Table 8: Iron status of mice on different diets used for lymphocyte-transformation experiments performed in serum-free medium.

| Diet   | Normal             |                    | Semisynthetic<br>+ Fe (SD) |                    | Semisynthetic<br>- Fe (DD) |                    | Paired<br>(PF)     |                    |
|--|--------------------|--------------------|----------------------------|--------------------|----------------------------|--------------------|--------------------|--------------------|
|  | I <sup>†</sup>     | II <sup>†</sup>    | I                          | II                 | I                          | II                 | I                  | II                 |
| Liver iron <sup>+</sup><br>(µg/g)                | 2.15 ± 0.08<br>(5) | 2.57 ± 0.08<br>(2) | 1.86 ± 0.15<br>(5)         | 2.42 ± 0.30<br>(2) | 0.54 ± 0.04<br>(5)         | 0.59 ± 0.05<br>(2) | 2.10 ± 0.19<br>(5) | 2.40 ± 0.30<br>(2) |
| Serum iron <sup>+</sup><br>(µg/100 ml)           | 238 ± 21<br>(5)    | 239 ± 10<br>(2)    | 274 ± 28<br>(5)            | 223 ± 15<br>(2)    | 51 ± 6<br>(5)              | 59 ± 18<br>(2)     | 238 ± 15<br>(5)    | 227 ± 12<br>(2)    |
| TIBC <sup>+</sup><br>(µg/100 ml)                 | 288 ± 2            | 290 ± 0            | 322 ± 4                    | 286 ± 5            | 490 ± 20                   | 425 ± 5            | 300 ± 0            | 310 ± 5            |
| % Saturation<br>of trans-<br>ferrin with<br>iron | 83                 | 82                 | 85                         | 78                 | 10                         | 14                 | 79                 | 73                 |

Figures in parentheses are number of animals. For statistical analysis, see Table 9.

<sup>+</sup> Mean ± SEM

<sup>†</sup> Two experiments performed, I and II, using two sets of mice.

**Table 2:** Analysis by Scheffé's method of multiple comparisons of the results of iron status of mice on different diets used for lymphocyte-transformation experiments performed in serum-free medium (data from Table 8).

| Groups compared* | LIVER IRON                         |                        |                                    |                        | SERUM IRON                         |                        |                                    |                        |
|------------------|------------------------------------|------------------------|------------------------------------|------------------------|------------------------------------|------------------------|------------------------------------|------------------------|
|                  | I†                                 |                        | II†                                |                        | I                                  |                        | II                                 |                        |
|                  | Simultaneous 95% confidence limits | Significant difference | Simultaneous 95% confidence limits | Significant difference | Simultaneous 95% confidence limits | Significant difference | Simultaneous 95% confidence limits | Significant difference |
| 1 and 2          | 0.87,-0.25                         | ns                     | 1.41,-1.11                         | ns                     | 5.96,-79.16                        | ns                     | 54.77,-22.77                       | ns                     |
| 1 and 3          | 2.18, 1.02                         | S                      | 3.24,0.72                          | S                      | 229.26,144.14                      | S                      | 218.77,141.23                      | S                      |
| 1 and 4          | 0.63,-0.08                         | ns                     | 1.43,-1.09                         | ns                     | 42.16,-42.96                       | ns                     | 50.77,-26.77                       | ns                     |
| 2 and 3          | 1.90,0.74                          | S                      | 3.09,0.57                          | S                      | 183.17,263.43                      | S                      | 202.77,125.23                      | S                      |
| 2 and 4          | 0.34,-0.84                         | ns                     | 1.28,-1.24                         | ns                     | 79.33,-3.93                        | ns                     | 34.77,-42.77                       | ns                     |
| 3 and 4          | -0.98,-2.14                        | S                      | -0.55,-3.07                        | S                      | -145.97,-227.23                    | S                      | -129.23,-206.77                    | S                      |

\* 1,2,3,4: mice fed on ND, SD, DO and PF, respectively.

† Two experiments performed, I and II, using two sets of mice.



transferrin compared with other groups of mice (Table 10), the difference was not significant (Table 11) and the effect was not reproducible in the subsequent experiments. The iron content of the medium, therefore, did not affect the cells of the DD group very differently from those of the other groups (Tables 10 and 11). This indicated that the lymphocytes from the iron-deficient mice responded normally to ConA and had not lost their ability to transform due to intrinsic defects caused by iron deficiency. Statistical analysis also showed that there was a day-to-day variation probably as a result of minor changes in the time of the day during which the experiments were performed or variations in the mice, and cell populations during each experiment (Table 11). The day-to-day variation might have caused the large standard deviations seen in Table 10, so it was possible that some small intrinsic difference in lymphocyte function in the DD group might not have been apparent. Since preliminary studies (not reported here) have shown that media containing FCS generally gave less variation in the mitogenic response than serum-free medium the experiment was repeated using medium containing FCS as well as serum-free medium containing transferrin.

#### 3.4.2.2. Transformation of lymphocytes in serum-free medium or in medium containing foetal calf serum

##### 3.4.2.2.1. Iron status

The serum and liver iron levels and the TIBC of mice (Table 12) were similar to the previous groups of mice (Table 8).

**Table 10: Transformation of lymphocytes from mice on different diets cultured with concanavalin A in serum-free medium.**

| $^{14}\text{C}$ -thymidine incorporation (c.p.m.) |                |                 |                 |                            |                 |                            |                |                 |                 |
|---|----------------|-----------------|-----------------|----------------------------|-----------------|----------------------------|----------------|-----------------|-----------------|
| Diet  | $\dagger$<br>I | Normal<br>(ND)  | $\dagger$<br>II | Semisynthetic<br>+ Fe (SD) |                 | Semisynthetic<br>- Fe (DD) |                | Paired<br>(PF)  |                 |
|   |                |                 |                 | I                          | II              | I                          | II             | I               | II              |
| Medium<br>serum-free <sup>+</sup><br>+ 10% Felf   | +ConA*         | 2029 $\pm$ 1165 | 2598 $\pm$ 1011 | 1689 $\pm$ 1360            | 2836 $\pm$ 1598 | 1107 $\pm$ 934             | 1083 $\pm$ 378 | 1661 $\pm$ 1258 | 1853 $\pm$ 554  |
|   | -ConA*         | 98 $\pm$ 32     | 177 $\pm$ 74    | 52 $\pm$ 19                | 203 $\pm$ 126   | 67 $\pm$ 31                | 160 $\pm$ 8    | 67 $\pm$ 22     | 168 $\pm$ 30    |
| serum-free <sup>+</sup><br>+ 30% Felf             | +ConA          | 5338 $\pm$ 2917 | 6731 $\pm$ 3357 | 4732 $\pm$ 3557            | 7750 $\pm$ 1313 | 4265 $\pm$ 3406            | 6196 $\pm$ 116 | 3277 $\pm$ 1881 | 5828 $\pm$ 3577 |
|   | -ConA          | 103 $\pm$ 34    | 221 $\pm$ 41    | 74 $\pm$ 42                | 270 $\pm$ 57    | 81 $\pm$ 51                | 234 $\pm$ 33   | 76 $\pm$ 43     | 158 $\pm$ 110   |

For statistical analysis see Table 11.

$\dagger$  Mean  $\pm$  SD.

$\dagger$  Two experiments performed, I and II, using two sets of mice.

\* n = 5 mice (Exp. I), or 2 mice (Exp. II), 7 observations/mouse for +ConA; 4 observations/mouse for -ConA.

Table 11: Three-way analysis of variance of the mitogenic response of lymphocytes from mice on different diets cultured in serum-free medium (data from Table 10).

| Due to                         | F ratio |       | Significant difference |             |
|--------------------------------|---------|-------|------------------------|-------------|
|                                | I       | II    | I                      | II          |
| Day-to-day variation           | 8.87    | 0.73  | $p < 0.001$            | ns          |
| Diet                           | 1.12    | 0.61  | ns                     | ns          |
| Medium                         | 29.26   | 21.31 | $p < 0.001$            | $p < 0.001$ |
| Interaction of medium and diet | 0.50    | 0.09  | ns                     | ns          |

#### 3.4.2.2.2. Mitogenic responses of lymphocytes

As before, the degree of mitogenic response of lymphocytes in serum-free medium containing 10%-iron-saturated transferrin was very much lower in all groups compared with the proliferation of cells cultured in medium containing 30%-iron-saturated transferrin or 10% FCS (Table 14). Furthermore, no significant difference in transformation was observed between cells cultured in media containing 30%-iron-saturated transferrin or FCS (Tables 14 and 15). The iron content of FCS was 1.89  $\mu\text{g/ml}$  and the saturation of serum transferrin with iron was 41%. Although the experiment using FCS in the culture medium was performed to account for the large variation in the proliferative activity of lymphocytes cultured in serum-free medium, the variation still persisted when cells were cultured in either medium. This result indicated that variation in mice or cell population was probably the cause of the large difference in lymphocyte proliferating activity rather than the serum-free conditions. Furthermore, three-way ANOVA (Table 15) showed that there was a slight but statistically significant effect of diet ( $p < 0.05$ ). However, this effect could have been a false positive since there was no significant effect when the interaction of medium and diet was considered, and furthermore, the effect of diet did not persist when the results of "30%  $\text{FeT}_f$ " versus "10% FCS" were analysed (Table 15).

#### 3.4.3. Effect of mouse serum on the transformation of mouse lymphocytes

Table 12: Iron status of mice on different diets used for lymphocyte-transformation experiments performed in serum-free medium or in medium containing foetal calf serum.

| Diet   | Normal<br>(ND)         | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>-Fe (DD) | Pairfed<br>(PF)        |
|--|------------------------|----------------------------|---------------------------|------------------------|
| Liver iron <sup>+</sup><br>( $\mu$ g/g)          | $2.44 \pm 0.27$<br>(5) | $2.26 \pm 0.13$<br>(5)     | $0.68 \pm 0.07$<br>(5)    | $2.52 \pm 0.36$<br>(5) |
| Serum iron <sup>+</sup><br>( $\mu$ g/100 ml)     | $230 \pm 23$<br>(5)    | $246 \pm 20$<br>(5)        | $84 \pm 9$<br>(5)         | $280 \pm 21$<br>(5)    |
| TIBC <sup>+</sup><br>( $\mu$ g/100 ml)           | $285 \pm 5$            | $280 \pm 2$                | $375 \pm 5$               | $300 \pm 10$           |
| % saturation<br>of trans-<br>ferrin with<br>iron | 81                     | 88                         | 22                        | 93                     |

Figures in parentheses are number of animals. For statistical analysis see Table 13.

<sup>+</sup>Mean  $\pm$  SEM.

**Table 13:** Analysis by Scheffé's method of multiple comparisons of the results of iron status of mice on different diets used for lymphocyte-transformation experiments performed in serum-free medium or in medium containing foetal calf serum (data from Table 12).

| Groups compared* | LIVER IRON                         |                        | SERUM IRON                         |                        |
|------------------|------------------------------------|------------------------|------------------------------------|------------------------|
|                  | Simultaneous 95% confidence limits | Significant difference | Simultaneous 95% confidence limits | Significant difference |
| 1 and 2          | 0.71,-0.35                         | ns                     | 66.85,-99.45                       | ns                     |
| 1 and 3          | 1.23,0.70                          | S                      | 229.15,62.85                       | S                      |
| 1 and 4          | 0.45,-0.61                         | ns                     | 33.15,-133.15                      | ns                     |
| 2 and 3          | 2.11,1.05                          | S                      | 245.45,79.15                       | S                      |
| 2 and 4          | 0.27,-0.79                         | ns                     | 49.45,-116.85                      | ns                     |
| 3 and 4          | -1.31,-2.37                        | S                      | -112.85,-279.15                    | S                      |

\*1,2,3,4: mice fed on ND, SD, DD and PF, respectively.

**Table 14:** Transformation of lymphocytes from mice on different diets cultured with concanavalin A in serum-free medium or in medium containing foetal calf serum.

| <sup>14</sup> C-thymidine incorporation (c.p.m.) |                                 |                            |                            |                        |  |
|--|---------------------------------|----------------------------|----------------------------|------------------------|--|
| Diet   | Normal<br>(ND)                  | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>- Fe (DD) | Paired<br>(PF)         |  |
| Medium   |                                 |                            |                            |                        |  |
| Serum-free <sup>+</sup><br>+ 10% FcFf            | +ConA*<br>1632 <sup>+</sup> 425 | 1161 <sup>+</sup> 550      | 836 <sup>+</sup> 99        | 1814 <sup>+</sup> 1020 |  |
|  | -ConA*<br>135 <sup>+</sup> 63   | 99 <sup>+</sup> 42         | 102 <sup>+</sup> 34        | 126 <sup>+</sup> 72    |  |
| Serum-free <sup>+</sup><br>+ 30% FcFf            | +ConA<br>3916 <sup>+</sup> 410  | 3564 <sup>+</sup> 686      | 3590 <sup>+</sup> 318      | 4122 <sup>+</sup> 1210 |  |
|  | -ConA<br>186 <sup>+</sup> 76    | 142 <sup>+</sup> 95        | 154 <sup>+</sup> 38        | 229 <sup>+</sup> 47    |  |
| 10% FCS <sup>+</sup>                             | +ConA<br>3438 <sup>+</sup> 1115 | 3118 <sup>+</sup> 1020     | 3971 <sup>+</sup> 757      | 3733 <sup>+</sup> 921  |  |
|  | -ConA<br>710 <sup>+</sup> 424   | 502 <sup>+</sup> 436       | 525 <sup>+</sup> 375       | 727 <sup>+</sup> 443   |  |

For statistical analysis, see Table 15.

<sup>+</sup> Mean  $\pm$  SD.

\* n = 5 mice, 7 observations/mouse for +ConA; 4 observations/mouse for -ConA.

Table 15: Three-way analysis of variance of the mitogenic response of lymphocytes from mice on different diets cultured in serum-free medium or in medium containing foetal calf serum (data from Table 14).

|               | of 10% FeTf vs 30% FeTf |                        | of 30% FeTf vs 10% FCS |                        |
|---------------|-------------------------|------------------------|------------------------|------------------------|
|               | F ratio                 | Significant difference | F ratio                | Significant difference |
| Due to        |                         |                        |                        |                        |
| Day-to-day    |                         |                        |                        |                        |
| variation     | 3.37                    | p < 0.05               | 0.93                   | ns                     |
| Diet          | 4.06                    | p < 0.05               | 1.13                   | ns                     |
| Medium        | 158.00                  | p < 0.001              | 1.27                   | ns                     |
| Interaction   |                         |                        |                        |                        |
| of medium and |                         |                        |                        |                        |
| diet          | 0.47                    | ns                     | 0.08                   | ns                     |



#### 3.4.3.1. Preliminary experiments

It has been reported (W. Haston, personal communications) that the mitogenic responses of mouse lymphocytes cultured in autologous serum for approximately three days is very low. In these experiments, different concentrations of ConA and times for culturing the cells were, therefore, used in order to determine whether different conditions from those used in previous experiments were necessary for optimum transformation. Pooled cells of the 14- to 15-week old mice of the same litter and pooled mouse sera which had been heat-inactivated (56°C for 10 min) were used in each experiment. Since medium containing either 2.5% or 5% mouse serum allowed the same degree of transformation (Table 16), medium containing 2.5% mouse serum was used in subsequent experiments. Also, there was no significant difference in the mitogenic response when cells were cultured for 44 or 54 h (Tables 17 and 18) and, therefore, the cells were cultured for 44 h in the subsequent experiments. Similarly, medium containing 1 µg/ml ConA did not induce as good proliferative response as 2 or 4 µg/ml ConA was used (Tables 17 and 18). Since there was no significant difference in the mitogenic when 2 or 4 µg/ml of ConA were used (Table 18), ConA was used at concentrations of 2 µg/ml, in the subsequent experiments.

#### 3.4.3.2. Effect of iron-sufficient, iron-deficient and iron-supplemented iron-deficient mouse sera on the transformation of mouse lymphocytes

Table 16: The effect of mouse serum on the transformation of mouse lymphocytes.

| Culture medium containing     | <sup>14</sup> C-thymidine incorporation (c.p.m.) |        |
|-------------------------------|--|--------|
|                               | +ConA*   | -ConA* |
| 2.5% mouse serum <sup>†</sup> | 2963 ± 300 <sup>‡</sup>                          | 66 ± 9 |
| 5% mouse serum <sup>†</sup>   | 2865 ± 221 <sup>‡</sup>                          | 69 ± 9 |

Cells cultured for 56 h.

<sup>†</sup> Mean ± SD.

\*+ConA, 4 µg/ml; n = 14; -ConA, n = 8.

<sup>‡</sup> p > 0.1.

**Table 17:** Transformation of mouse lymphocytes cultured at different times with different concentrations of concanavalin A in medium containing 2.5% mouse serum.

| $^{14}\text{C}$ -thymidine incorporation (c.p.m.) |                    |                              |                    |             |
|---|--------------------|------------------------------|--------------------|-------------|
|   | 1 $\mu\text{g/ml}$ | +ConA*<br>2 $\mu\text{g/ml}$ | 4 $\mu\text{g/ml}$ | -ConA*      |
| Culturing period <sup>†</sup> (h)                 |                    |                              |                    |             |
| 30  | 2523 $\pm$ 371     | 3084 $\pm$ 74                | 3045 $\pm$ 128     | 27 $\pm$ 6  |
| 44  | 3299 $\pm$ 568     | 4245 $\pm$ 211               | 4712 $\pm$ 704     | 34 $\pm$ 9  |
| 54  | 3347 $\pm$ 394     | 3923 $\pm$ 409               | 4695 $\pm$ 352     | 39 $\pm$ 10 |

For statistical analysis, see Table 18.

<sup>†</sup> Mean  $\pm$  SD.

\*+ConA, n = 10; -ConA, n = 6.

Table 18: Analysis by Scheffé's method of multiple comparisons of results of the effect of different concentrations of concanavalin A and culturing time on the transformation of mouse lymphocytes (data from Table 17).

| Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|------------------|------------------------------------|------------------------|
| 1 and 2          | -232.4, -889.6                     | S                      |
| 1 and 3          | -193.4, -850.6                     | S                      |
| 2 and 3          | 367.6, -289.6                      | ns                     |
| 4 and 5          | -162.4, -1729.6                    | S                      |
| 4 and 6          | -629.4, -2196.6                    | S                      |
| 5 and 6          | 316.6, -1250.6                     | ns                     |
| 7 and 8          | -2.0, -1150.0                      | S                      |
| 7 and 9          | -174.0, -1322.0                    | S                      |
| 8 and 9          | 402.0, -746.0                      | ns                     |
| 1 and 4          | -126.0, -1426.0                    | S                      |
| 1 and 7          | -174.0, -1474.0                    | S                      |
| 4 and 7          | 602.0, -698.0                      | ns                     |
| 2 and 5          | -740.2, -1581.8                    | S                      |
| 2 and 8          | -418.2, -1259.8                    | S                      |
| 5 and 8          | 742.8, -98.8                       | ns                     |
| 3 and 6          | -1311.0, -2023.0                   | S                      |
| 3 and 9          | -1294.0, -2006.0                   | S                      |
| 6 and 9          | 373.0, -339.0                      | ns                     |

\*1,4,7: 1 µg/ml ConA; 2,5,8: 2 µg/ml ConA; 3,6,9: 4 µg/ml ConA.  
 1,2,3: cells cultured for 30 h; 4,5,6: cells cultured for 44 h;  
 7,8,9: cells cultured for 54 h.

Since the previous experiments showed no significant difference between any control groups with respect to either iron status (see 3.4.2.1.1. and 3.4.2.2.1) or lymphocyte transformation (3.4.2.1.2. and 3.4.2.2.2) the ND and PF groups were omitted in this experiment. Mice were therefore fed on either iron-containing semisynthetic diet (SD; 10 mice) or low-iron diet (DD; 10 mice) as described in Section 3.3.1.

#### 3.4.3.2.1. Iron status

Since mouse serum was used in the culture medium and as inadequate amounts of serum could be obtained from each mouse to perform all assays individually, serum iron and TIBC were estimated using pooled sera of all the mice of the same group. Again, the serum iron levels of the DD group were much lower and the TIBC much higher than the SD group (Table 19). Liver iron was estimated in all mice individually, and was significantly lower in the DD group than the SD group (Table 19). Again the results indicated that mice fed on low-iron diet were iron deficient.

#### 3.4.3.2.2. Mitogenic response of lymphocytes

Two mice of each group were killed per day over a period of five consecutive days. Sera and lymph-node cells of mice of the same groups killed each day were pooled. Different combinations of "cells" - "Serum" were used; cells from mice fed on iron-containing semisynthetic diet (SD) were cultured in media containing:

Table 19: Iron status of mice on different diets used for lymphocyte-transformation experiments performed in medium containing mouse serum.

| Diet   | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>- Fe (DD) |
|--|----------------------------|----------------------------|
| Liver*<br>( $\mu\text{g/g}$ )                          | $3.24 \pm 0.32$            | $0.68 \pm 0.12$            |
| Serum <sup>+</sup><br>( $\mu\text{g}/100 \text{ ml}$ ) | $289 \pm 7$                | $108 \pm 3$                |
| TIBC <sup>+</sup><br>( $\mu\text{g}/100 \text{ ml}$ )  | $330 \pm 10$               | $520 \pm 2$                |
| % Saturation<br>of trans-<br>ferrin with<br>iron       | 88                         | 21                         |

\*p < 0.001; Mean of readings from 10 mice; 2 readings/mouse  $\pm$  SEM.

<sup>+</sup>Mean of two readings of pooled sera from 10 mice  $\pm$  SEM.

1. iron-sufficient mouse serum (SS)
2. iron-deficient mouse serum (DS)
3. iron-deficient mouse serum supplemented with 1.8  $\mu\text{g}/\text{ml}$  iron (DS + Fe).

Similarly, cells from mice fed on low-iron diet (DD) were cultured in media containing SS or DS or DS + Fe. Addition of iron to DS serum was based on the difference between the average level of iron in the iron-deficient mouse sera and in the sera from the control groups of mice calculated from Tables 8 and 12. This was found to be 172  $\mu\text{g}/100$  ml serum which was rounded off to 180  $\mu\text{g}/100$  ml and it was added as Fe(NTA) solution. Cells from iron-sufficient and iron-deficient mice were also cultured with ConA (1  $\mu\text{g}/\text{ml}$ ) in serum-free media containing either 10%-- or 30%-iron-saturated transferrin.

Transformation of lymphocytes from iron-sufficient or iron-deficient mice was lower in media containing serum from iron-deficient mice than when serum from iron-sufficient mice was used (Tables 20 and 21). Addition of iron to the deficient serum significantly improved its ability to promote transformation. These results again show that the lymphocytes from iron-deficient mice have a normal capacity to transform in response to mitogens, and in addition demonstrated that serum from iron-deficient mice has inadequate iron levels to permit optimum in vitro transformation. However, although deficient serum with added iron was as good as iron-sufficient serum in supporting transformation of lymphocytes from iron-deficient mice, it was still somewhat

**Table 20:** Effect of iron-sufficient, iron-deficient mouse sera and iron-deficient mouse serum supplemented with iron on the transformation of lymphocytes from mice on different diets.

| $^{14}\text{C}$ -thymidine incorporation (c.p.m.)  |                            |                            |
|--|----------------------------|----------------------------|
| Diet   | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>- Fe (DD) |
| Medium supplemented<br>with <sup>+</sup>   |                            |                            |
| Iron-sufficient<br>mouse serum (SS) +ConA*   | 5569 $\pm$ 377             | 5068 $\pm$ 777             |
| -ConA*   | 44 $\pm$ 23                | 62 $\pm$ 32                |
| Iron-deficient<br>mouse serum (DS) +ConA   | 3655 $\pm$ 601             | 3752 $\pm$ 601             |
| -ConA  | 51 $\pm$ 22                | 55 $\pm$ 30                |
| Iron-deficient<br>mouse serum<br>supplemented<br>with 1.8 $\mu\text{g/ml}$<br>Fe (DS + Fe) +ConA | 4619 $\pm$ 622             | 4969 $\pm$ 846             |
| -ConA  | 44 $\pm$ 18                | 45 $\pm$ 10                |

For statistical analysis, see Table 21.

<sup>+</sup> Mean  $\pm$  SD.

\* n = 10 mice, 7 observations/mouse for +ConA; 4 observations/mouse for -ConA.



**Table 21:** Three-way analysis of variance of the mitogenic response of lymphocytes from mice on different diets cultured in medium containing iron-sufficient or iron-deficient mouse serum or iron-deficient mouse serum supplemented with iron (data from Table 20).

|                                | Excluding iron-sufficient serum |                        | Excluding iron-deficient serum |                        | Including all data |                        |
|--------------------------------|---------------------------------|------------------------|--------------------------------|------------------------|--------------------|------------------------|
|                                | F ratio                         | Significant difference | F ratio                        | Significant difference | F ratio            | Significant difference |
| Due to                         |                                 |                        |                                |                        |                    |                        |
| Day-to-day variation           | 21.97                           | $p < 0.001$            | 30.52                          | $p < 0.001$            | 37.00              | $p < 0.001$            |
| Diet                           | 3.59                            | ns                     | 0.89                           | ns                     | 0.15               | ns                     |
| Medium                         | 106.65                          | $p < 0.001$            | 26.30                          | $p < 0.001$            | 134.94             | $p < 0.001$            |
| Interaction of medium and diet | 1.10                            | ns                     | 17.06                          | $p < 0.001$            | 9.84               | $p < 0.001$            |

inferior when used with cells from iron-sufficient mice (Table 20). This indicates that serum from iron-deficient mice may lack other growth factors unrelated to iron content.

The mitogenic response of the cells from these mice was also tested using serum-free conditions, and as before, proliferation was significantly lower in medium containing 10%-iron-saturated transferrin when compared with the responses in media supplemented with 30%-iron-saturated transferrin (Table 22). Again, no significant difference was observed between responses of cells from iron-sufficient and iron-deficient mice (Tables 22 and 23).

#### 3.4.4. Protein synthesis by transformed lymph-node lymphocytes from mice fed on different diets

In the previous experiments it was found that the iron-content of the culture medium is important in controlling the mitogenic responses of mouse lymphocytes. The aim of these experiments was to find whether the iron level in the culture medium also affects protein synthesis by lymphocytes in vitro.

Twenty mice were fed on diets as described in 3.3.1, and lymph-node lymphocytes were cultured in serum-free medium containing 10%-or-30% iron-saturated transferrin (see 2.3.2). Protein synthesis by transformed cells was determined as described elsewhere (see 3.3.9). Two experiments were performed using two different sets of four groups of mice, one group for each diet (ND, SD, DD, PF). In the first experiment the transformed lymphocytes were

Table 22: Transformation of lymphocytes from mice on different diets cultured with concanavalin A in serum-free medium.

| $^{14}\text{C}$ -thymidine incorporation (c.p.m.) |        |                            |                           |
|---|--------|----------------------------|---------------------------|
| Diet  |        | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>-Fe (DD) |
| Medium supplemented<br>with <sup>+</sup>          |        |                            |                           |
| 10% FeTf  | +ConA* | 1818 $\pm$ 425             | 1793 $\pm$ 410            |
|   | -ConA* | 161 $\pm$ 53               | 110 $\pm$ 15              |
| 30% FeTf  | +ConA  | 3539 $\pm$ 212             | 3279 $\pm$ 640            |
|   | -ConA  | 187 $\pm$ 97               | 150 $\pm$ 23              |

For statistical analysis see Table 23.

<sup>+</sup> Mean  $\pm$  SD.

\* n = 10 mice, 3 observations/mouse for +ConA; 2 observations/mouse for -ConA.

Table 23: Analysis by Scheffé's method of multiple comparisons of the results of transformation of lymphocytes from mice on different diets cultured with concanavalin A in serum-free medium (data from Table 22).

| Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|------------------|------------------------------------|------------------------|
| 1 and 2          | 328.7,-278.7                       | ns                     |
| 1 and 3          | -1417.3,-2024.7                    | S                      |
| 3 and 4          | 563.7,-43.7                        | ns                     |
| 2 and 4          | -1182.3,-1789.7                    | S                      |

\*1,3: cells from mice fed on SD diet and cultured in medium containing 10% FeTf or 30% FeTf, respectively.

2,4: cells from mice fed on DD diet and cultured with 10% FeTf or 30% FeTf, respectively.

washed and protein synthesis determined in media containing FCS. However, in the second experiment protein synthesis was assessed in medium containing 10%-or 30%-iron-saturated transferrin as appropriate.

3.4.4.1. Experiment I: transformed cells cultured in media containing foetal calf serum

3.4.4.1.1. Iron status

Haemoglobin levels were estimated on this occasion to determine whether the iron-deficient group of mice develop anaemia in addition to iron deficiency under the feeding conditions employed throughout this work. Since inadequate amounts of blood could be obtained from each mouse by heart puncture to permit other assays to be carried out as well, serum iron and TIBC were not estimated in this group. The haemoglobin levels of the iron-deficient group were lower but not statistically different from the other groups (Tables 24(a) and 25). These results indicate that the mice of the DD group were not anaemic. The liver iron levels of the DD group were significantly lower than iron levels of the other groups (Tables 24(a) and 25). Furthermore, the liver iron values were similar to those in previous experiments (Tables 8, 12, 19), and it was assumed that serum iron levels would also have been similar. Since the mice were not anaemic, but the low liver iron levels show that a state of latent iron deficiency existed.

Table 24(a): Iron status of mice on different diets used for estimating protein synthesis by transformed lymphocytes in medium containing foetal calf serum.

| Diet                               | Normal<br>(ND)         | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>-Fe (DD) | Paired<br>(PF)         |
|------------------------------------|------------------------|----------------------------|---------------------------|------------------------|
| Hb <sup>+</sup><br>(g /100 ml)     | 13.5 $\pm$ 0.5<br>(5)  | 14.5 $\pm$ 0.6<br>(5)      | 11.4 $\pm$ 1.1<br>(5)     | 13.8 $\pm$ 0.9<br>(5)  |
| Liver <sup>+</sup><br>( $\mu$ g/g) | 3.20 $\pm$ 0.43<br>(5) | 2.47 $\pm$ 0.23<br>(5)     | 0.70 $\pm$ 0.04<br>(5)    | 2.30 $\pm$ 0.29<br>(5) |

Figures in parentheses are number of animals. For statistical analysis, see Table 25.

<sup>+</sup> Mean  $\pm$  SEM.

Figure 24(b): Iron status of mice on different diets used for estimating protein synthesis by transformed lymphocytes in medium containing transferrin.

| Diet                               | Normal<br>(ND)         | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>- Fe (DD) | Paired<br>(PF)         |
|------------------------------------|------------------------|----------------------------|----------------------------|------------------------|
| Hb <sup>+</sup><br>(g /100 ml)     | 13.0 $\pm$ 0.6<br>(5)  | 13.6 $\pm$ 0.7<br>(4)      | 7.4 $\pm$ 0.5<br>(5)       | 12.6 $\pm$ 0.4<br>(5)  |
| Liver <sup>+</sup><br>( $\mu$ g/g) | 2.70 $\pm$ 0.22<br>(5) | 2.30 $\pm$ 0.34<br>(4)     | 0.66 $\pm$ 0.01<br>(5)     | 2.89 $\pm$ 0.20<br>(5) |

Figures in parentheses are number of animals. For statistical analysis, see Table 25.

<sup>+</sup>Mean  $\pm$  SEM.

Table 25: Analysis by Scheffé's method of multiple comparisons of results of the iron status of mice on different diets used for estimating protein synthesis (data from Tables 24a and 24b).

| Groups compared* | LIVER IRON  |   |   | HAEMOGLOBIN  |  |  |
|------------------|---|---|---|--|--|--|
|                  | <sup>a+</sup><br>Simultaneous<br>95% confidence<br>limits | <sup>a+</sup><br>Signific-<br>ant diff-<br>erence | <sup>b+</sup><br>Simultaneous<br>95% confidence<br>limits | <sup>a</sup><br>Simultaneous<br>95% confidence<br>limits | <sup>a</sup><br>Signific-<br>ant diff-<br>erence | <sup>b</sup><br>Simultaneous<br>95% confidence<br>limits |
| 1 and 2          | 2.00,-0.54  | ns  | 1.50,-0.70  | 2.59,-4.69   | ns   | 2.00,-3.07   |
| 1 and 3          | 3.75,1.24   | S   | 3.12,0.92   | 5.70,-1.58   | ns   | 8.00,3.26  |
| 1 and 4          | 2.16,-0.36  | ns  | 0.83,-1.25  | 3.29,-4.00   | ns   | 2.74,-2.00   |
| 2 and 3          | 3.03,0.51   | S   | 2.72,0.52   | 6.75,-0.53   | ns   | 8.34,3.30  |
| 2 and 4          | 1.44,-1.08  | ns  | 0.44,-1.71  | 4.33,-2.94   | ns   | 3.44,-1.60   |
| 3 and 4          | -0.34,-2.85   | S   | -1.19,-3.27   | 1.23,-6.05   | ns   | -2.89,-7.63  |

\*1,2,3,4: mice fed on ND, SD, DD and PF, respectively.

+ a,b: transformed lymphocytes cultured in medium containing FCS and transferrin, respectively.



#### 3.4.4.1.2. Protein synthesis

Estimation of protein synthesis by lymphocytes which had been transformed in serum-free medium containing 10%- or 30%-iron-saturated transferrin revealed no significant difference between any group of mice (Table 26(a)). It appeared, therefore, that iron deficiency or iron-availability in the medium did not affect protein synthesis by transformed lymphocytes. However, since the transformed lymphocytes were washed and cultured in media containing FCS, the iron level in the FCS might have been large enough to eliminate any effect on protein synthesis of the low-iron content of the medium to which apotransferrin was added. The experiment was, therefore, repeated but the transformed lymphocytes were washed and cultured in medium to which apotransferrin or 20%-iron-saturated transferrin were added as appropriate.

#### 3.4.4.2. Experiment III: transformed cells cultured in media containing transferrin

For technical reasons, the mice in this experiment were fed on the different diets for seven weeks instead of six weeks as in previous experiments.

##### 3.4.4.2.1. Iron status

The haemoglobin level of the iron-deficient group was significantly lower compared with the levels in any other group (Table 24(b)), indicating that the mice of the iron-deficient group had developed anaemia. It appears, therefore, that an extra week of feeding on low-iron diet may have been long enough to convert the latent iron deficiency state into anaemia.

Table 26(a): Protein synthesis by transformed lymphocytes estimated in medium containing foetal calf serum.

|   | <sup>3</sup> H-leucine incorporation (c.p.m.) |                    |                            |                            |                    |
|---|---|--------------------|----------------------------|----------------------------|--------------------|
|   | Diet  | Normal<br>(ND)     | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>- Fe (DD) | Paired<br>(PF)     |
| Lymphocytes transformed in serum-free medium containing 10% FeTf <sup>+</sup> |   | 4926 ± 986<br>(5)  | 4921 ± 1763<br>(4)         | 3347 ± 931<br>(5)          | 3716 ± 2059<br>(4) |
| 30% FeTf <sup>+</sup>   |   | 5859 ± 2040<br>(5) | 4771 ± 748<br>(4)          | 3662 ± 1802<br>(5)         | 5696 ± 799<br>(4)  |

Figures in parentheses are number of animals.

Three-way ANOVA: 10% FeTf vs 30% FeTf: day-to-day variation,  $F = 1.19$  (ns); diet,  $F = 2.51$  (ns); medium,  $F = 1.99$  (ns). Interaction: medium and diet,  $F = 0.85$  (ns).

<sup>+</sup>Mean  $\pm$  SD.

The liver iron in the DD group was significantly lower compared with the other groups (Tables 24(b) and 25).

#### 3.4.4.2.2. Protein synthesis

Lymphocytes from any group of mice transformed in serum-free medium containing either 10%-or 30%-iron-saturated transferrin synthesized similar levels of protein (Table 26(b)). These results confirmed the earlier observation that neither iron deficiency nor iron availability in the culture medium affects protein synthesis by transformed lymphocytes indicating that once DNA has been synthesized translation and protein synthesis proceed normally irrespective of iron availability.

#### 3.4.5. Effect of *in vivo* repletion of iron-deficient mice on lymphocyte transformation *in vitro*

The aim of these experiments was to determine whether repletion of mice fed on low-iron diet restores lymphocyte responses to normal values and whether this is associated with re-establishment of normal iron status, particularly serum iron levels.

##### 3.4.5.1. Preliminary experiments

Since Kuvibidila et al. (1983a) had shown that iron repletion could be established within two weeks, four mice were fed on low-iron diet for six weeks after which the mice were put on iron-supplemented diet for three weeks to determine whether this was long enough to restore iron levels. Another group of four mice were fed on iron-deficient diet for the period of the nine weeks.

Table 26(b): Protein synthesis by transformed lymphocytes estimated in medium containing transferrin.

|   | <sup>3</sup> H-leucine incorporation (c.p.m.) |                     |                            |                            |                    |
|---|---|---------------------|----------------------------|----------------------------|--------------------|
|   | Diet  | Normal<br>(ND)      | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>- Fe (DD) | Paired<br>(PF)     |
| Lymphocytes transformed in serum-free medium containing |   |                     |                            |                            |                    |
| 10% FeTf <sup>+</sup>                                   |   | 10648 ± 3230<br>(5) | 8699 ± 1385<br>(4)         | 8852 ± 1642<br>(4)         | 7347 ± 3326<br>(5) |
| 30% FeTf <sup>+</sup>                                   |   | 10156 ± 780<br>(5)  | 9183 ± 888<br>(4)          | 9604 ± 1138<br>(4)         | 8722 ± 1593<br>(4) |

Figures in parentheses are number of animals.

Three-way ANOVA: 10% FeTf vs 30% FeTf: day-to-day variation, F = 0.69 (ns); diet, F = 0.28 (ns); medium, F = 0.61 (ns). Interaction: medium and diet, F = 0.28 (ns).

<sup>+</sup>Mean ± SD.

At the end of the feeding period, haemoglobin levels and serum and liver iron were estimated. The iron status of the iron-deficient group (serum iron:  $75 \pm 11 \mu\text{g}/100 \text{ ml}$ ; liver iron:  $0.80 \pm 0.04 \mu\text{g}/\text{g}$ ; haemoglobin:  $7.2 \pm 0.5 \text{ g} / 100 \text{ ml}$ ; mean  $\pm$  SEM) was similar to values obtained for the DD group in the previous experiments, and in the iron-repleted mice, the serum iron ( $230 \pm 18 \mu\text{g}/100 \text{ ml}$ ) and liver iron ( $2.2 \pm 0.3 \mu\text{g}/\text{g}$ ) were similar to values obtained for the ND, SD and PF groups in the previous experiments. The haemoglobin levels of the iron-repleted mice ( $11.4 \pm 1.4 \text{ g} / 100 \text{ ml}$ ) were very near to figures obtained in Section 3.4.5.1.1. These results indicated that, as found by Kuvibidila et al. (1983a), iron repletion had been established in iron-deficient mice after feeding on iron-supplemented diet for three weeks. However since haemoglobin levels were slightly below normal values obtained in Sections 3.4.4.1.1. and 3.4.4.2.1, the iron deficient mice were fed on iron-supplemented diet for four weeks, in the subsequent experiments.

3.4.5.2. The effect of serum from iron-sufficient, iron-repleted and iron-deficient mice on the transformation of mouse lymphocytes in vitro

Nine mice were assigned to three groups (three mice per group) and fed on different diets as described in Section 3.3.2. After the feeding period the mice were killed (see 3.3.3).

#### 3.4.5.2.1. Iron status

The serum and liver iron levels of the repleted group of mice were very much the same as that of the group fed on iron-containing semisynthetic diet (Table 27), while there was a significant difference between the repleted mice and the iron-deficient group (Tables 27 and 28). These results confirmed the observations of the preliminary experiment (see 3.4.5.1) that supplementation of iron in the low-iron diet brings the liver and serum iron levels of the iron-deficient mice back to normal values.

#### 3.4.5.2.2. Mitogenic responses of mouse lymphocytes from iron-sufficient, iron-deficient and iron-repleted mice

Lymph-node lymphocytes were cultured for 43 h in medium containing 2.5% mouse serum and 1 µg/ml ConA (batch 2). Cells from iron-repleted mice were cultured in serum from iron-sufficient, iron-deficient and iron-repleted mice and also in iron-deficient serum supplemented with iron and serum from repleted mice was used to test cells from all three groups. Additions of iron to DS serum was carried out as described in Section 3.4.3.2.2. Cultures containing cells from deficient mice in iron-deficient serum, and cells from mice fed on iron-supplemented diet in normal serum were also set up. The transformation of lymphocytes from iron-deficient or iron-repleted mice was lower in media supplemented with serum from iron-deficient mice than when

**Table 27:** Iron status of iron-sufficient, iron-repleted and iron-deficient mice.

| Mice                                   | Iron-sufficient | Iron-repleted   | Iron-deficient  |
|--|-----------------|-----------------|-----------------|
| Serum iron <sup>+</sup><br>(µg/100 ml) | 229 ± 29 (3)    | 220 ± 10 (3)    | 70 ± 6 (3)      |
| Liver iron <sup>+</sup><br>(µg/g)      | 2.31 ± 0.30 (3) | 2.40 ± 0.26 (3) | 0.52 ± 0.03 (3) |

Figures in parentheses are number of animals. For statistical analysis, see Table 28.

<sup>+</sup>Mean ± SEM.

**Table 28:** Analysis by Scheffé's method of multiple comparisons of results of the iron status of mice used for iron-repletion studies (data from Table 27).

| Groups compared* | LIVER IRON                         |                            | SERUM IRON                         |                        |
|------------------|------------------------------------|----------------------------|------------------------------------|------------------------|
|                  | Simultaneous 95% confidence limits | Signifi- cant diff- erence | Simultaneous 95% confidence limits | Significant difference |
| 1 and 2          | 0.14,-0.05                         | ns                         | 72.80,-86.80                       | ns                     |
| 1 and 3          | 0.21,0.09                          | S                          | 248.80,89.25                       | S                      |
| 2 and 3          | 0.25,0.05                          | S                          | 255.83,96.21                       | S                      |

\*1: iron-sufficient mice; 2: iron-repleted mice; 3: iron-deficient.

serum from iron-sufficient or iron-repleted mice was used (Table 29). As before, addition of sufficient iron to bring the iron level of the deficient serum to that of iron-sufficient serum significantly improved its ability to promote transformation. Cells from iron-repleted mice, transformed as well as the cells from normal mice, and serum from iron-repleted mice was as good as serum from iron-sufficient mice and/or iron-supplemented iron-deficient serum in promoting transformation. The degree of transformation of lymphocytes was, however, generally reduced in comparison with previous experiments (Table 20), this probably being associated with changing the batch of ConA. The results presented here indicated that repletion of iron deficiency brings about a restoration of normal lymphocyte responses to ConA, this being the result of an increase in serum iron levels, and confirm the earlier observations that iron deficiency in mice does not cause intrinsic defects in lymphocytes associated with ability to transform.

#### 3.4.6. Mouse T-cell populations and subpopulations

In the previous experiments it was shown that iron deficiency affects T-lymphocyte transformation in vitro, this being associated with a decrease in the level of circulating iron. However, it might be possible that iron deficiency affects immune responses by altering the relative populations of T-lymphocyte populations and subpopulations. In order to find whether this was true, experiments were performed to study T-cell and T-cell subsets in populations of cells from mouse lymph nodes.



**Table 29:** Effect of sera from iron-sufficient, iron-deficient and iron-repleted mice on the transformation of mouse lymphocytes.

| Medium supplemented with serum from                | <sup>14</sup> C-thymidine incorporation (c.p.m.) |                 |                                 |
|--|--|-----------------|---------------------------------|
|  | Cells from mice                                  | iron-sufficient | iron-deficient iron-repleted    |
| Iron-sufficient mice                               |  | 1976 ± 191 (19) | nd 2100 ± 275 (19)              |
| Iron-deficient mice                                |  | nd              | 1111 ± 315 (19) 1043 ± 129 (19) |
| Iron-repleted mice                                 |  | 2200 ± 447 (19) | 1882 ± 492 (19) 1909 ± 417 (19) |
| Iron-deficient mice supplemented with 1.8 µg/ml Fe |  | nd              | nd 1946 ± 506 (18)              |

Figures in parentheses are number of observations (6-7 per mouse). Mean ± SD.

Two-way ANOVA: day-to-day variation and source effect (i.e. effect of choosing eight different combinations of cell and serum) were highly significant ( $F = 23.68$ ,  $p < 0.001$ ;  $F = 35.64$ ,  $p < 0.001$  respectively). The differences between all possible pairs of eight source combinations were analysed by Scheffé's method of multiple comparisons: combination of DS-cell significantly different from all other combinations. No significant difference among any other groups.

3.4.6.1. Estimation of T-lymphocyte populations using ANAE stain

Twenty mice were assigned to four groups and fed on different diets as described in Section 3.3.1. Iron status and the percentage of ANAE-positive T-cells of the four groups of mice are shown in Tables 30 and 34, respectively. The liver iron levels (Table 30) were similar to values obtained from previous groups of mice (Tables 8, 12, 19) and furthermore, the haemoglobin levels were similar to values obtained in Section 3.4.4.1.1, thus confirming the observation that when mice are fed on low-iron diets for six weeks, the haemoglobin levels do not fall much below those of the control groups. The percentage of T-cells in the iron-deficient group was not statistically different from that of the other groups of mice (Tables 34 and 35).

3.4.6.2. Estimation of T-lymphocyte population and sub-populations using monoclonal antibody to T-cell surface antigens

Using fluorescein-conjugated  $\alpha$ -thy<sub>1,2</sub>,  $\alpha$ -ly<sub>1</sub> and  $\alpha$ -ly<sub>2</sub> monoclonal antibodies the relative proportions of T-cells and T-cell subsets in the lymph nodes of mice fed on ND, SD, DD, and PF was studied. Figure 8 shows an example of lymph-node cells positive for thy<sub>1,2</sub> antigen. Similar results were obtained with  $\alpha$ -ly<sub>1</sub> and  $\alpha$ -ly<sub>2</sub> antibodies. As before, liver and serum iron levels in the iron-deficient group were significantly lower than in control groups (Tables 32 and 33). The proportion of cells positive for

Table 30: Iron status of mice on different diets used for T-cell studies with  $\alpha$ -naphthyl acetate esterase stain.

| Diet                                    | Normal<br>(ND)      | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>- Fe (DD) | Paired<br>(PF)      |
|---|---------------------|----------------------------|----------------------------|---------------------|
| Hb levels <sup>+</sup><br>(g /100 ml)   | 12.5 $\pm$ 0.9 (5)  | 13.0 $\pm$ 0.6 (5)         | 11.4 $\pm$ 0.4 (5)         | 13.6 $\pm$ 0.6 (5)  |
| Liver iron <sup>+</sup><br>( $\mu$ g/g) | 2.87 $\pm$ 0.96 (5) | 2.54 $\pm$ 0.20 (5)        | 0.63 $\pm$ 0.04 (5)        | 2.22 $\pm$ 0.20 (5) |

Figures in parentheses are number of animals. For statistical analysis, see Table 31.

<sup>+</sup> Mean  $\pm$  SEM.

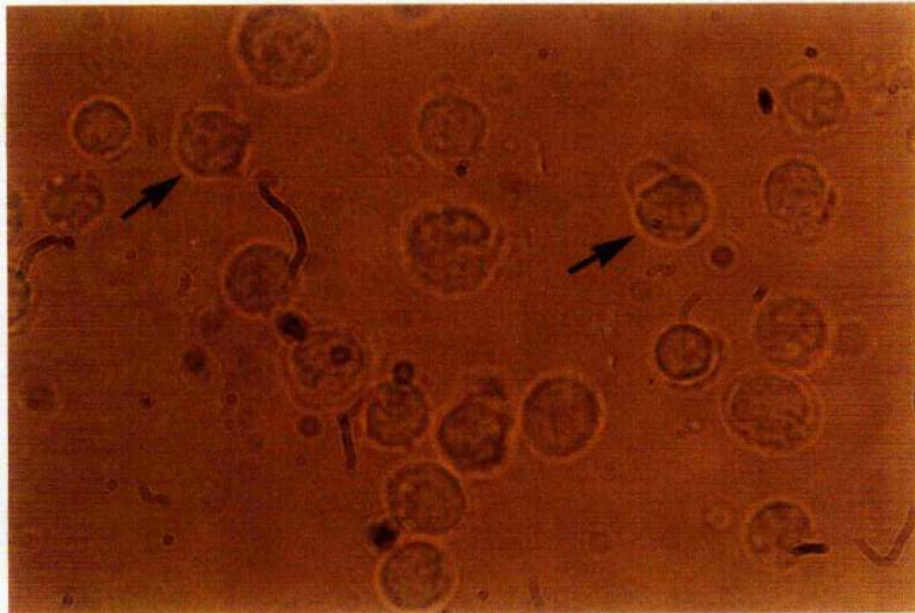
**Table 31:** Analysis by Scheffe's method of multiple comparisons of results of the iron status of mice on different diets used for T-cell studies with  $\alpha$ -naphthyl acetate esterase stain (data from Table 30).

| Groups compared* | HAEMOGLOBIN                        |                        | LIVER IRON                         |                        |
|------------------|------------------------------------|------------------------|------------------------------------|------------------------|
|                  | Simultaneous 95% confidence limits | Significant difference | Simultaneous 95% confidence limits | Significant difference |
| 1 and 2          | 3.33,-2.33                         | ns                     | 1.15,-0.49                         | ns                     |
| 1 and 3          | 3.93,-1.73                         | ns                     | 3.06,1.43                          | S                      |
| 1 and 4          | 1.73,-3.93                         | ns                     | 1.47,-0.17                         | ns                     |
| 2 and 3          | 4.43,-3.43                         | ns                     | 2.73,1.10                          | S                      |
| 2 and 4          | 2.23,-3.43                         | ns                     | 1.14,-0.50                         | ns                     |
| 3 and 4          | 0.63,-5.03                         | ns                     | -0.78,-2.41                        | S                      |

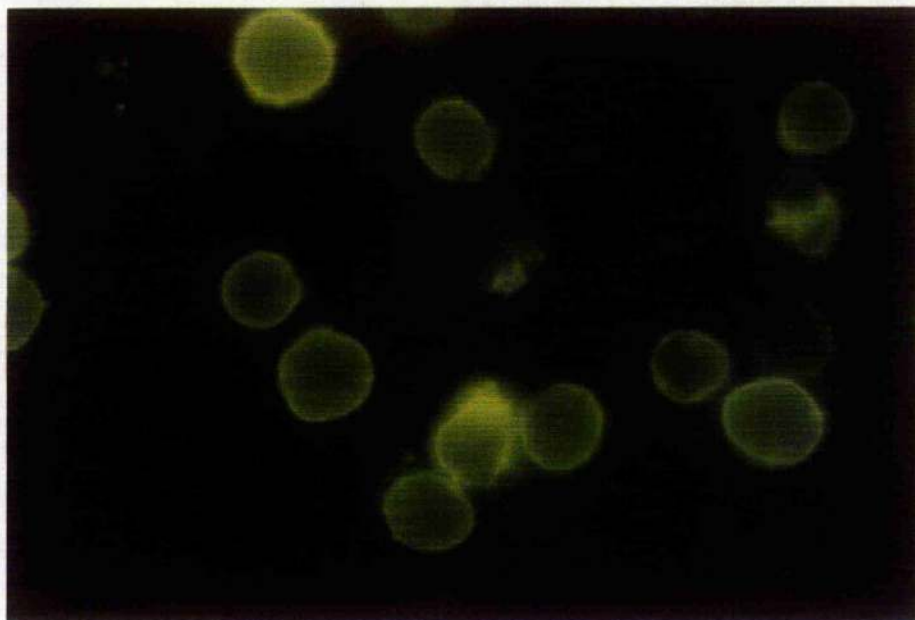
\*1,2,3,4: mice fed on ND, SD, DD and PF, respectively.

Figure 8: A population of cells from mesenteric and peripheral lymph nodes of mice stained with monoclonal antibody to thy<sub>1,2</sub> antigen.

(a)



(b)



(a) Cells as seen under phase contrast ( $\times 1300$ )

(b) The same field as in (a) under U.V., showing thy<sub>1,2</sub> positive cells (i.e. T-lymphocytes).  
The arrows in (a) point to non-T-lymphocytes which have not stained with the fluorescent antibody.

Table 32: Iron status of mice on different diets used for T-cell and T-cell subset studies with monoclonal antibody to T-cell antigens.

| Diet                                   | Normal<br>(ND)     | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>- Fe (DD) |
|--|--------------------|----------------------------|----------------------------|
| Serum iron <sup>+</sup><br>(µg/100 ml) | 197 ± 32<br>(3)    | 219 ± 18<br>(4)            | 55 ± 7<br>(4)              |
| Liver iron <sup>+</sup><br>(µg/g)      | 1.80 ± 0.80<br>(3) | 2.02 ± 0.07<br>(4)         | 0.51 ± 0.03<br>(4)         |

Figures in parentheses are number of animals. For statistical analysis, see Table 33.

<sup>+</sup>Mean ± SEM.

Table 33: Analysis by Scheffé's method of multiple comparisons of the iron status of mice on different diets used for T-cell studies with monoclonal antibody to T-cell antigens. (Data from Table 32).

| Groups compared* | SERUM IRON                               |                           | LIVER IRON                               |                           |
|------------------|--|---------------------------|--|---------------------------|
|                  | Simultaneous<br>95% confidence<br>limits | Significant<br>difference | Simultaneous<br>95% confidence<br>limits | Significant<br>difference |
| 1 and 2          | 60.14,-104.14                            | ns                        | 0.09,-0.08                               | ns                        |
| 1 and 3          | 59.86,224.14                             | S                         | 0.16, 0.13                               | S                         |
| 2 and 3          | 238.17,85.83                             | S                         | 0.19, 0.15                               | S                         |

\*1,2,3: mice fed on ND, SD and DD, respectively.

Table 34: T-cells and T-cell subsets in the lymph nodes of mice on different diets.

| Diet  | Normal<br>(ND) | Semisynthetic<br>+ Fe (SD) | Semisynthetic<br>- Fe (DB) | Paired<br>(PF) |
|---|----------------|----------------------------|----------------------------|----------------|
| Percentage T-cells +ve<br>with ANAE-stain <sup>†</sup>  | 85 ± 3 (5)     | 87 ± 2 (5)                 | 86 ± 4 (5)                 | 85 ± 3 (5)     |
| Percentage cells +ve for<br>thy <sub>1,2</sub> cell antigen <sup>†</sup>                              | 77.0 ± 1.2 (3) | 77.2 ± 0.5 (4)             | 76.5 ± 2.1 (4)             | nd             |
| Percentage of thy <sub>1,2</sub> -<br>positive cells,<br>+ve for ly <sub>1</sub> antigen <sup>†</sup> | 83.4 ± 0.5 (3) | 81.6 ± 0.5 (4)             | 76.7 ± 1.9 (4)             | nd             |
| Percentage of thy <sub>1,2</sub> -<br>positive cells,<br>+ve for ly <sub>2</sub> antigen <sup>†</sup> | 33.4 ± 0.5 (3) | 34.1 ± 0.3 (4)             | 33.6 ± 1.0 (4)             | nd             |

Figures in parentheses are number of animals. For statistical analysis, see Table 35.

<sup>†</sup>Mean ± SEM.

**Table 35:** Analysis by Scheffé's method of multiple comparisons of results obtained from studies on T-cell and T-cell subsets in mouse lymph nodes (data from Table 34).

| Groups compared* | Simultaneous 95% confidence limits |                               |                               |                             | Significant difference           |                               |                               |                             |
|------------------|------------------------------------|-------------------------------|-------------------------------|-----------------------------|----------------------------------|-------------------------------|-------------------------------|-----------------------------|
|                  | Cells +ve for thy <sub>1,2</sub>   | Cells +ve for ly <sub>1</sub> | Cells +ve for ly <sub>2</sub> | T-cells +ve with ANAE-stain | Cells +ve for thy <sub>1,2</sub> | Cells +ve for ly <sub>1</sub> | Cells +ve for ly <sub>2</sub> | T-cells +ve with ANAE-stain |
| 1 and 2          | 6.66,-6.66                         | 10.35,-8.35                   | 3.81,-5.81                    | 4.39,-8.39                  | ns                               | ns                            | ns                            | ns                          |
| 1 and 3          | 7.66,-5.66                         | 15.35,-3.35                   | 4.81,-4.81                    | 5.39,-7.39                  | ns                               | ns                            | ns                            | ns                          |
| 2 and 3          | 7.16,-5.16                         | 13.65,-3.65                   | 5.45,-3.45                    | 6.39,-6.39                  | ns                               | ns                            | ns                            | ns                          |
| 1 and 4          | nd                                 | nd                            | nd                            | 7.39,-5.39                  | nd                               | nd                            | nd                            | ns                          |
| 2 and 4          | nd                                 | nd                            | nd                            | 8.39,-4.39                  | nd                               | nd                            | nd                            | ns                          |
| 3 and 4          | nd                                 | nd                            | nd                            | 7.39,-5.39                  | nd                               | nd                            | nd                            | ns                          |

\*1,2,3,4: mice fed on ND, SD, DD and PF, respectively.



thy<sub>1,2</sub>, ly<sub>1</sub> and ly<sub>2</sub> did not differ significantly between any groups of mice (Tables 34 and 35). This suggested that iron deficiency did not affect the mitogenic responses of T-lymphocytes by altering the relative proportions of different T-cell subpopulations.

### 3.5 DISCUSSION

Iron is an important requirement for metabolically active cells especially those undergoing differentiation and cell division, it being needed in particular for the synthesis of iron-containing proteins and enzymes involved in DNA synthesis. Lymphocyte transformation, a process of elevated metabolic activity leading to cell division, may also be associated with an increased requirement for iron.

Decreased lymphocyte proliferative responses in vitro in association with iron deficiency have been reported by many investigators using culture media supplemented with either autologous serum (Joynson et al., 1972; Fletcher et al., 1975) or foetal calf serum (Macdougall et al., 1975; Sawitsky et al., 1976; Soyano et al., 1982; Kuvibidila et al., 1983a,b). However, other authors have reported no effect of iron deficiency on the mitogenic response of lymphocytes using either autologous plasma (Kulapongs et al., 1974) or normal human serum (Gross et al., 1975; Gupta et al., 1982; Krantman et al., 1982) in the culture medium. This controversy is probably due, at least in part, to lack of an experimental system which allows accurate control of iron supply during the in vitro culture of lymphocytes.

The work reported in this chapter has attempted to define more closely the effect of iron deficiency on lymphocyte transformation in vitro using an experimental system in which the iron content of the culture medium was accurately monitored, taking advantage of the fact that

mouse lymphocytes can respond to ConA in serum-free medium (Brock, 1981). Furthermore, the use of an animal model has allowed variables which could complicate the interpretation of the results such as infections and other concomitant nutritional deficiencies to be eliminated.

In the present study, the mean weight-gain of mice fed on different semisynthetic diets was independent of the iron content of the diet or the amount consumed per day even when there was a day-to-day variation. However, the average weight-gain of the mice fed on iron-containing semisynthetic diet was significantly higher compared with that when normal laboratory diet was used (Fig. 7, Table 5). This may have been due to the fact that the semisynthetic diet had a higher carbohydrate content (64.8%) than the laboratory diet (48.4%). Furthermore, although the mean weight-gain of mice fed on low-iron diet was slightly lower than that of the mice fed on iron supplemented diet or paired this difference was not statistically significant (Fig. 7, Table 6). These results differ from the observations of Kuvibidila *et al.* (1983a) who at the end of their 32-day feeding period found a statistically significant reduction in the mean body weight of the iron-deficient mice and paired group in comparison with the control group. However, this difference was observed in 25% of their animals only, as 75% of the mice of each group had already been killed by this time, and they did not find a significant difference in the mean weight-gain of any mice during the first 25 days of feeding. They also used mice that were of a different strain and considerably older than those used in this study.

A 6-week feeding period on low-iron diet was sufficient to induce iron deficiency in mice as indicated by the low serum and liver iron levels ( $< 95 \mu\text{g}/100 \text{ ml}$  and  $< 0.75 \mu\text{g}/\text{g}$ , respectively) compared with values obtained from the control group ( $> 180 \mu\text{g}/100 \text{ ml}$  and  $> 1.8 \mu\text{g}/\text{g}$ , respectively). These results are comparable with values obtained by other investigators (Puschmann & Ganzoni, 1977; Kuvibidila *et al.*, 1983a,b). At the end of the 6-week feeding period, the haemoglobin levels of the iron-deficient group were lower, but not to a statistically significant degree than those of the control mice. The low liver iron and near normal haemoglobin level in the iron-deficient groups (Tables 24(a) and 30) indicated that latent iron deficiency had been established. However, after seven weeks of feeding on the low-iron diet, a statistically significant reduction in the haemoglobin levels in the iron-deficient group was observed (Table 24(b)). These results indicate that after 7 weeks on low-iron diet the mice become anaemic. However, although anaemia was not observed after 6-week feeding period on low-iron diet there was little difference in the level of liver iron between mice fed on low-iron diet for six weeks and those fed for seven weeks (see Tables 24(a) and 24(b)), so mice were normally fed for six weeks before being used.

As in the preliminary experiments (Chapter 2), the degree of transformation of lymphocytes from mice cultured in medium containing 10%-iron-saturated transferrin was significantly lower compared with the responses of the cells cultured in medium containing 30%-iron-saturated

transferrin or FCS. This applied to all groups, irrespective of diet and iron status. This result indicates that one of the important factors in controlling the proliferative responses of lymphocytes in vitro is the content of transferrin-bound iron in the culture medium. When transferrin partially saturated with iron is added to the culture medium, the amount of iron taken up by cells from transferrin and hence the degree of cell proliferation, will depend on the percentage of apo, monoferric and diferric species of transferrin bound to the transferrin receptor on the cell membrane. The proportion of each species of transferrin bound will depend, in turn, on the saturation of transferrin with iron (Williams et al., 1978). With rabbit reticulocytes, apotransferrin binds to the transferrin receptor with lower affinity than mono- and diferric transferrins, and their binding affinities are  $4.6 \times 10^6 \text{ M}^{-1}$ ,  $2.65 \times 10^7 \text{ M}^{-1}$  and  $1.1 \times 10^8 \text{ M}^{-1}$ , respectively (Young et al., 1984b). At low saturation of transferrin with iron, therefore, the concentration of apotransferrin may be sufficiently high to counteract its relatively low binding affinity for the transferrin receptor. This could then lead to a significant blocking of receptors by apotransferrin and hence reduced iron uptake from the iron containing molecules. The proportion of apotransferrin, monoferric and diferric species of transferrin bound by the cells can be calculated using the affinity constants for these species as estimated for reticulocytes by Young et al. (see above), together with the values for the distribution of iron between the binding

sites in partially saturated transferrin as determined by Williams et al. (1978). The percentage of a particular species of transferrin expected to bind to the transferrin receptor is equal to: proportion of the species present multiplied by its affinity constant for the transferrin receptor. Thus, for 10%-iron-saturated transferrin, the proportion of apotransferrin, monoferric and diferric transferrin which would be expected to bind to the receptor are 31, 41 and 28% respectively, in comparison with 8, 55 and 37%, respectively, when 30%-iron-saturated transferrin is used. Thus, with 10 %-iron-saturated transferrin the proportion of apotransferrin bound would be high in comparison with that bound when transferrin 30% saturated with iron is used, and as a result, the interference by apotransferrin in the iron uptake process will be higher. If these calculations are applied to the present study it can be proposed that transferrin at low iron saturations would not be as good an iron donor to cells as transferrin of higher iron saturation, and hence the lymphocyte proliferative responses would be reduced. Furthermore, although foetal calf serum containing medium had a higher saturation of (bovine) transferrin with iron (41%; see 3.4.2.2.2) than the serum-free medium, the degree of transformation in both media was not significantly different (Table 14) indicating that under the conditions employed here, culture medium containing 30%-iron-saturated transferrin is as good as medium containing 10% FCS in supporting and promoting lymphocyte transformation.

Using serum from iron-sufficient and iron-deficient mice, it was also shown that sera from iron-deficient mice were unable to support optimum transformation of cells isolated either from iron-deficient mice or control mice, while serum from iron-sufficient mice permitted a normal response of lymphocytes from both iron-deficient and iron-sufficient mice. Supplementation of iron deficient sera with enough iron to bring the iron levels back to normal values (i.e. to increase the saturation of serum transferrin with iron) increased the lymphocyte mitogenic responses. It seems, therefore, that the decreased lymphocyte proliferation associated with iron deficiency may be due to inadequate levels of circulating transferrin-bound iron. As previously explained, the apotransferrin present in the serum will interfere with the uptake of iron by the cells from monoferric and diferric species of transferrin. The average percentage of saturation of transferrin with iron in the iron-deficient and iron-sufficient sera as calculated from Tables 8 and 12, is 15% and 82%, respectively. At 15% saturation of transferrin with iron, the theoretical proportions of apotransferrin, monoferric and diferric transferrins bound would be 19%, 52% and 29%, respectively. When transferrin is 82% saturated with iron most of the transferrin bound would be in the diferric form (91.8%) and only 0.3% in the apo form. Therefore, in medium containing serum from normal mice, there is a large amount of iron available to the cells, this being the result of low

interference from apotransferrin and also due to the higher proportion of diferric to monoferric transferrin bound to the cells.

The observation that the degree of lymphocyte proliferation depends on the availability of transferrin-bound iron is supported by the work of other investigators. It has been shown that lymphocytes isolated from iron-deficient individuals responded normally to mitogens when cultured in medium containing serum from normal individuals (Gross et al., 1975; Van Heerden et al., 1981; Krantman et al., 1982). Normal human serum contains approximately 2.4 mg/ml transferrin (Morgan, 1981) which is about one third saturated with iron (Fairbanks & Beutler, 1977) so that when serum is added to the lymphocyte culture medium at a normal concentration of 5-10%, there will, as explained above, be enough circulating transferrin-bound iron to permit a normal response of lymphocytes to mitogens (or antigens). Furthermore, the normal response to mitogens of lymphocytes isolated from iron-deficient subjects and cultured in medium containing serum from normal individuals also indicated that iron deficiency did not cause any intrinsic defects in lymphocytes and that these cells can respond normally if there is enough circulating transferrin-bound iron.

Decreased proliferative responses of lymphocytes associated with reduced levels of circulating transferrin-bound iron were also reported by Joynson et al. (1972), who cultured peripheral blood cells isolated from iron deficient anaemic patients in a medium containing autologous



serum. The level of circulating iron in the deficient group ( $25.5 \pm 3.68 \mu\text{g}/100 \text{ ml}$ ) was much lower than that of the normal individuals ( $115.3 \pm 7.57 \mu\text{g}/100 \text{ ml}$ ). The reduced transformation observed by the above investigators might therefore have been associated with decreased levels of circulating transferrin-bound iron present in the culture medium. However, they also cultured cells in medium containing 20% foetal bovine serum, and as it was <sup>not</sup> specified in the results which of the two types of serum was used, it is not clear whether the decreased lymphocyte transformation was the result of low iron levels in the culture medium. Decreased lymphocyte proliferation in vitro not associated with decreased levels of transferrin-bound iron in the culture medium have also been reported. Kuvibidila et al. (1983b) showed that the response to ConA of spleen cells from iron deficient mice cultured in medium containing foetal calf serum was significantly lower than that of the control groups and that this difference was not eliminated by addition of ferric chloride or mouse transferrin (unspecified saturation with iron) to the culture medium. Their results indicated that the low lymphocyte proliferative response was probably the result of cell abnormalities caused as a result of iron deficiency, rather than the lack of adequate levels of transferrin-bound iron. However, it is difficult to compare their results with those presented here as spleen rather than lymph node cells were used. There was also an unexplained variation in the

effect of in vitro iron repletion according to the mitogen and source of iron employed. Fletcher et al. (1975) also observed decreased proliferation of lymphocytes isolated from iron-deficient individuals and cultured in medium containing plasma from iron deficient patients. The decreased response, though, was not due to low-levels of iron in the culture medium but probably due to intrinsic defects of the cells isolated from the iron-deficient patients, since cells from normal subjects cultured in "iron-deficient" serum produced a normal response. However, most of their patients had chronic candida infections which might have affected parameters associated with lymphocyte function. On the other hand, it has also been reported that lymphocytes isolated from iron-deficient patients and cultured in medium containing autologous plasma could transform normally in response to mitogens, indicating that neither iron-deficiency nor availability of transferrin-bound iron affected lymphocyte transformation (Kulapongs et al., 1974). However, all their patients had a concomitant hookworm infection, which may alter cell-mediated immune responses or enhance the production of factors which could balance out the negative effect of lack of iron. It should be noted that reports in the literature indicate that factors other than iron in the culture medium may affect lymphocyte transformation. The presence of infection (Mendes and Raphael, 1971) influences cell-mediated immune responses and nutritional restriction in the diet causes damage of the lymphoid system and substantially alters the

immune response (Faulk et al., 1975; Malavé et al., 1980). The probable reason for the demonstration by many investigators of a reduced mitogenic or antigenic response of lymphocytes isolated from iron-deficient individuals and cultured in media without restriction of iron may have been the presence of infections or nutritional deficiencies other than iron (Bhaskaram & Reddy, 1975; Chandra & Saraya, 1975; Macdougall et al., 1975; Sawitsky et al., 1976). It is important to note that investigators who failed to find abnormalities in lymphocyte transformation associated with iron deficiency using culture medium containing serum from healthy individuals were among those who documented the absence of infections or nutritional factors other than iron (Gross et al., 1975; Gupta et al., 1982; Krantman et al., 1982).

Furthermore, using sera from normal, iron-repleted and iron-deficient mice it was shown that sera from iron-sufficient mice were able to support optimum transformation of lymphocytes isolated from iron-deficient, iron-sufficient or iron-repleted mice, while serum from iron-deficient mice could not (Tables 20 and 29). The observed increase in proliferative activity of lymphocytes cultured in medium containing serum from iron-repleted mice was probably the result of the in vivo restoration of normal levels of circulating transferrin-bound iron. The finding that decreased skin responses of iron-deficient mice (Kuvibidila et al., 1981) or iron-deficient patients (Talwalkar et al., 1982) were restored to normal values by iron administration,

may also be explained by increased levels of circulating transferrin-bound iron.

Another observation in the present investigation was that the iron content of the medium did not affect the cells of the iron-deficient group differently from those of the other groups of mice. Cells from iron-deficient mice would transform as well as cells from iron-sufficient mice in all media. These results indicate that iron deficiency in mice does not cause any intrinsic defects in lymphocytes which affect proliferation and differentiation in vitro. This conclusion is supported by the work of Cummins et al. (1978) who showed that immune lymph node cells obtained either from normal or iron-deficient donor rats failed to induce parasite rejection in iron-deficient recipients, whereas immune lymphocytes from deficient donors could induce rejection in normal recipients. However, abnormalities in lymphocytes as a result of iron deficiency have been reported. Kuvibidila et al. (1983a) demonstrated a reduced blastogenic response of mouse splenic lymphocytes cultured with ConA or PHA in medium containing new born calf serum, which was restored to normal after in vivo iron repletion. They attributed the decreased mitogenic response to either increased numbers of undifferentiated or functionally inactive cells. Furthermore, Jarvis and Jacobs (1974) reported morphological abnormalities of the mitochondria, such as increase in size, degeneration of the cristae and vacuolation, in lymphocytes isolated from iron deficient and anaemic individuals. Similar observations were reported

by Jiménez et al. (1982) who in addition showed that the mitochondrial modifications were proportional to the degree of iron deficiency. It is possible, however, that the morphological abnormalities seen by these investigators may not affect transformation per se, or may only be found as a result of severe and prolonged iron deficiency, and do not develop in the comparatively short time for which the experimental animals were kept on low-iron diet in these experiments.

The proportion of T-lymphocytes in the mouse lymph nodes as indicated using monoclonal antibody to T-cell surface antigens and ANAE-stain was the same in normal and iron-deficient mice. This suggests that iron deficiency does not affect immune responses by altering the relative proportions of different lymphocyte subpopulations in the lymph nodes in mice. As iron deficiency affects cell division and differentiation it is to be expected that the population of lymphocytes with a high turnover rate (i.e. short-lived) would be more affected in iron deficiency than the long-lived cells. Since the lymph-node compartment is considered by represent a long-lived recirculating pool of lymphocytes (Zatz & Lance, 1970) such a difference in T-lymphocyte populations in lymph nodes in iron deficiency may not be present. In contrast to the above findings, Bhaskaram & Reddy (1975), Chandra & Saraya (1975), Srikantia et al. (1976) and Krantman et al. (1982) reported decreased T-cell counts associated with iron deficiency. However, they all used peripheral human blood cells while mouse

lymph node cells were used in the present study. Furthermore, a decreased proportion of T-cells in the spleens of iron deficient mice has also been reported (Kuvibidila et al., 1982; 1983a ). However the spleens in the iron-deficient mice were very much enlarged so that the absolute number of T-cells per spleen in the iron-deficient mice might not have been very much different from the control mice. In the present study the proportion of T-cells using ANAE-stain which was approximately 82-90% of the total lymph node cells (Table 34), was slightly lower (7-9%) than that demonstrated by Mueller et al. (1975). However, they used cells from mesenteric lymph nodes only while cells from mesenteric and peripheral lymph nodes were used in the present investigation and they also used BABL/c mice while C<sub>3</sub>H/Bi mice were used in this study. The proportion of cells positive for thy<sub>1,2</sub> cell-antigens was found to be about 76-78% (Table 34). This value was lower than that estimated using ANAE-stain. However, results obtained using ANAE-stain cannot be compared to those obtained using monoclonal antibody to T-cell surface antigens because ANAE-stain involves the detection of a cytoplasmic enzyme activity i.e. of a different cell marker to that detected by antibodies to T-cell surface antigens. The proportion of thy<sub>1,2</sub> positive cells which were also positive for either ly<sub>1</sub> or ly<sub>2</sub> cell surface antigens was approximately 74-83% and 32-34%, respectively, (Table 34), indicating that the number of cells positive for thy<sub>1,2</sub> (i.e. T-lymphocytes) is less than the number of cells positive for ly<sub>1</sub> (i.e.

T-helper) and  $ly_2$  (i.e. T-suppressor). However sensitive immunofluorescence techniques have shown that in mice  $ly_1$  is found in all T-cells although it is present in much higher levels on T-helper than on T-suppressor or cytotoxic T-cells (Ledbetter et al., 1980), so that some cells positive for  $ly_2$  may also be positive for  $ly_1$ .

Although DNA synthesis is affected by the amount of transferrin-bound iron in the culture medium, in the present study protein synthesis by transformed cells was not affected by the availability of iron. Resting lymphocytes, like all cells, possess the enzymatic apparatus required for oxidative metabolism and also other enzymes such as acid phosphatases and enzymes for the synthesis of DNA, RNA and protein (Elves, 1972). T-cell activation and proliferation leading to cell division, involves increased cellular metabolic activity such as DNA, RNA and protein synthesis (Epstein & Stolman, 1964; Inman & Cooper, 1965; Sell et al., 1965). Furthermore, it is well established that stimulated T-cells produce interleukin-2 (Smith, 1980; Smith et al., 1980b; Wagner et al., 1980), a T-cell growth factor which plays a crucial role in T-cell activation and proliferation and they also produce immune interferon (Perussia et al., 1980; Klein et al., 1982), a polypeptide involved in immunity against viruses. T-lymphocyte transformation is therefore associated with increased production of various proteins. The process of protein synthesis requires DNA and since transferrin-bound iron as seen in this study affects DNA-synthesis, it could be expected that availability of iron during proliferation

could also affect protein synthesis. However, from the results presented here, protein synthesis was not directly dependent on the levels of iron in the culture medium, and it seems, therefore, that iron deficiency did not cause any intrinsic defects in the ability of these cells to synthesise protein. It is important to note that under the conditions employed here for measuring total protein synthesis by transformed cells, the number of cells from each group was standardised to eliminate possible differences in protein synthesis arising from the presence of different number of cells. In general, it seems that, provided there is sufficient DNA to initiate translation, this and protein synthesis will proceed normally even in the condition of iron limitation.

In conclusion, a short term iron deficiency in mice does not affect the proliferative responses of mouse lymph node T-lymphocytes in vitro by causing intrinsic defects or by altering the relative proportion of different lymphocyte populations in the nodes. Furthermore, although protein synthesis seems to be independent of the iron level of the culture medium, cell transformation as measured by DNA synthesis depends on the availability of transferrin-bound iron. From the results presented in this chapter it is quite clear that proliferation of mouse lymph node T-lymphocytes in vitro depends on the availability of transferrin-bound iron. However, it was not evident whether transferrin was important in proliferation only as an iron donor or whether it also has an effect on cell transformation unrelated to its iron-donating properties.



In order to determine the exact role of transferrin in the proliferation and division of T-lymphocytes, another set of experiments was performed and these are discussed in more detail in Chapter 4.

### 3.6 CONCLUSIONS

1. No intrinsic loss of ability of mouse lymph-node T-cells to transform is caused as a result of iron deficiency.
2. The iron content of the culture medium is important for T-lymphocytes to transform in vitro.
3. The in vitro protein synthesis by transformed lymphocytes is not directly affected by the iron content of the culture medium.
4. Under the conditions employed, serum from iron-deficient mice has inadequate iron levels to permit optimum transformation.
5. In vivo iron repletion resulted in the re-establishment of normal iron status and also in restoration of the in vitro mitogenic response of lymphocytes cultured in medium containing serum from iron-repleted mice.
6. Iron deficiency does not affect the proportion of T-cells or T-cell subsets in the mesenteric and peripheral lymph nodes of mice.

### 3.7. SUMMARY

The in vitro response to ConA of lymphocytes from iron-deficient and control groups of mice in media containing either 10% FCS or 30%-iron-saturated transferrin was the same for the iron-deficient and control groups. However, the degree of transformation in serum-free medium containing 10%-iron-saturated transferrin was significantly lower in all groups of mice. The transformation of lymphocytes from iron-sufficient, iron-deficient and iron-repleted mice was lower in media supplemented with serum from iron-deficient mice than when serum from iron-sufficient or iron-repleted mice was used. Addition of sufficient iron to bring the iron level of the deficient serum to that of normal serum significantly improved its ability to promote transformation. In vivo repletion of iron-deficient mice resulted in a restoration of normal lymphocyte response to ConA which was associated with a return of serum iron levels to normal values. The proportion of cells positive for  $\text{thy}_{1,2}$ ,  $\text{ly}_1$  and  $\text{ly}_2$  antigens did not differ significantly between any groups of mice. Protein synthesis by cells transformed in serum-free medium containing 10%-or 30%-iron-saturated transferrin was the same in all groups of mice. These results indicate that the decreased lymphocyte-proliferative responses in iron deficiency may be due to inadequate levels of circulating transferrin-bound iron rather than to intrinsic defects caused as a result of iron deficiency or changes in the proportion of T-cells or T-cell subsets in the lymph nodes.

CHAPTER 4

THE ROLE OF TRANSFERRIN AND IRON  
IN PROMOTING THE IN VITRO TRANS-  
FORMATION OF MOUSE LYMPH NODE  
LYMPHOCYTES

#### 4.1. INTRODUCTION

Although iron is an important requirement for haemoglobin synthesis (Elder, 1980; Wrigglesworth & Baum, 1980), small but significant quantities also are required by cells other than erythroid precursors for the synthesis of metabolically important metalloenzymes and cytochromes (Wrigglesworth & Baum, 1980). The transformation of lymphocytes to form blast cells, an important component of the immune response, involves increased metabolic activity (MacKinney et al., 1962; Epstein & Stohlman, 1964; Inman & Cooper, 1965; Sell et al., 1965; Elves, 1972; Ling & Kay, 1975) and hence an increased requirement for iron-containing enzymes. It is possible, therefore, that inadequate delivery of iron may cause a decrease in the metabolic activity of lymphocytes which may result in reduced proliferative response.

In the previous chapter it was shown that lymphocyte transformation in serum-free or serum-containing medium, depended on the availability of transferrin-bound iron and that supplementation of iron-deficient serum with enough iron to bring the iron levels back to normal values significantly increased the degree of proliferation. Since it was not clear whether transferrin was important in transformation as an iron donor, or that transferrin also affects proliferation by means other than donating iron, it was decided to investigate further the role of transferrin in lymphocyte proliferation. The requirement of transferrin

and iron in lymphocyte transformation has been reported before. Tormey and Mueller (1972) showed increased incorporation of labelled thymidine into DNA in human peripheral blood lymphocytes when human transferrin was added to the culture medium. The interpretation of their results, though, is difficult since they cultured the lymphocytes in medium containing foetal calf serum and hence their results were complicated by the presence of bovine transferrin. Lipsky & Lietman (1980) showed that stimulated murine lymphocytes accumulated  $^{59}\text{Fe}$  during the blastogenic process when cultured in medium containing serum which had been incubated with  $^{59}\text{Fe}$  as ferrous chloride to allow oxidation and binding of the iron by the serum transferrin. Furthermore, mitogen-stimulated lymphocytes showed an enhanced incorporation of labelled thymidine when cultured with transferrin in serum-free medium (Phillips & Azari, 1975; Dillner-Centerlind *et al.*, 1979; Brock, 1981, Tanno *et al.*, 1982). This ability of transferrin to promote lymphocyte transformation *in vitro* was shown to depend on its ability to bind iron since desferrioxamine, an iron chelator, added to the medium prior to transferrin prevented transferrin from potentiating the proliferative responses (Brock, 1981; Novogrodsky *et al.*, 1982). These workers also showed that the decreased proliferative response of lymphocytes in the presence of desferrioxamine (DFO) was not the result of modification of the mitogenic response by desferrioxamine itself but probably due to removing transferrin and hence depriving

lymphocytes, of iron. Similarly, Lipsky & Lietman (1980) showed that the blastogenic response of murine lymphocytes cultured in serum-containing medium was decreased by about 50% when desferrioxamine was added to the medium. Additionally, they reported that the percentage inhibition depended on the time for which the cells had been cultured before desferrioxamine was added. Brock (1981) and Tanno et al. (1982) also reported that in serum-free conditions desferrioxamine inhibited the transformation-promoting activity of transferrin, and that the inhibition depended on the concentration of desferrioxamine.

Use of monoclonal antibodies to the transferrin receptor which blocks transferrin binding showed the need of transferrin-bound iron by proliferating cells in vitro. Transferrin-mediated uptake of iron by murine tumour cell lines was inhibited by an antibody to mouse transferrin receptor (Trowbridge et al., 1982). The inhibition of cell-proliferation by monoclonal antibody was not a direct result of its binding to the receptor, since a monoclonal antibody to transferrin receptor which did not block access of transferrin to the receptor did not have any effect (Trowbridge & Lopez, 1982). Further evidence that the transferrin requirement for in vitro lymphocyte proliferation is related to a need for transferrin-bound iron, comes from the observation that the degree of transformation of lymphocytes depends not only on the presence of transferrin, but also on the level of transferrin-bound iron (Phillips & Azari, 1975; Brock, 1981) indicating that the levels of

transferrin-bound iron are also important. This is confirmed by the work of Brock & Rankin (1981) and Homford et al. (1983), who showed that mitogen stimulation resulted in an increase in both binding of transferrin and uptake of iron by lymphocytes. Uptake of transferrin-bound iron and binding of transferrin by human lymphoblastoid cell lines has also been reported (Schulman et al., 1981). During transformation and cell division, when the requirement for iron increases, there is also an increased expression of cell-membrane receptors specific for transferrin (Hamilton et al., 1979; Larrick & Cresswell, 1979a; Galbraith et al., 1980a,b,c; Brock & Rankin, 1981; Hammarström et al., 1982; Neckers & Cossman, 1983).

Literature data, therefore, suggest that transferrin is important in lymphocyte proliferation as an iron supplier. However, it could also be proposed that the requirement for transferrin is not only related to the increased need of proliferating cells for iron, but may also result from cell proliferation being dependent upon a signal derived from the interaction of transferrin with its receptor. This would suggest that the importance of transferrin in lymphocyte transformation may be due to some effect on cell proliferation other than its ability to act as an iron donor. There are some literature data which support such a theory. Although iron from transferrin is important for lymphocyte proliferation, it is necessary to explain the fact that lymphocytes, in serum-free medium, also take iron from iron compounds such as nitrilotriacetate (Brock, 1981)



or ferrous sulphate (Tanno et al., 1982) but these compounds do not substitute for transferrin as promoters of lymphocyte transformation. However, it should be remembered that iron uptake from low molecular weight compounds is non-physiological since, under normal conditions, no low-molecular weight iron complexes are found in the plasma (Hahn & Ganzoni, 1980) and internal handling and utilisation is probably abnormal. Another point of consideration is that although desferrioxamine when added to cell cultures caused a marked inhibition of lymphocyte proliferation, it did not induce complete inhibition even at concentrations sufficient to chelate all the iron in the medium (Brock, 1981; Novogrodsky et al., 1982). Furthermore, proliferating lymphocytes in contrast to erythroid precursors bind inappropriately large amounts of transferrin in relation to their iron requirements (Bomford et al., 1983). Finally, lymphocytes possess storage iron (ferritin) which one might expect to be mobilised to meet increased needs during transformation if no exogenous iron is available, but seemingly ferritin cannot substitute for transferrin-bound iron (Summers & Jacobs, 1976). Taking these points into consideration, it could be argued that the binding of the iron-transferrin complex, or perhaps, transferrin alone, to the cell membrane receptor is, in itself, an important step in the sequence of events which lead to cell proliferation. Binding of transferrin to its receptor at physiological temperatures results in aggregation or patching of the receptors which is followed by endocytosis (Galbraith & Galbraith, 1980). Patching has been observed

in other systems such as the binding of mitogens to specific receptors on cell membrane (Ling & Kay, 1975; Stobo, 1977) or the binding of antigens to immunoglobulins on B-lymphocytes (Taylor et al., 1971; Loor et al., 1972; Raff & de Petris, 1973). The transferrin receptor is a major multi-subunit component in the membrane of transformed T-lymphocytes (Goding & Harris, 1981) and it is a bivalent molecule (Newman et al., 1982). Furthermore, transferrin probably acts as a divalent ligand since iron-binding half-molecules produce no enhancement of lymphocyte proliferative responses (Brock, 1981). Patching of the immunoglobulin on B-lymphocytes which leads to cell activation, also requires a divalent ligand (Taylor et al., 1971; Raff & de Petris, 1973). There is, therefore, a resemblance between the binding of transferrin to its receptor and other triggering systems such as the binding of antigen to surface immunoglobulin.

In conclusion, although transferrin is undoubtedly important for lymphocyte transformation as an iron donor, there are also data which suggest that the interaction of transferrin with its receptor on the membrane of stimulated cells may in itself trigger events leading to transformation. This study was performed in order to investigate this possibility, and four different approaches have been used to test this hypothesis.

1. The effect of desferrioxamine on the proliferation of mouse lymph node lymphocytes in vitro

One of the important factors which needs to be taken into account while investigating the effect of transferrin or iron on lymphocyte proliferation in vitro, is the level of endogenous iron or transferrin in the culture medium. The interpretation, for example, of results concerning the effect of transferrin or iron on the proliferation of lymphocytes cultured in serum-containing medium are complicated by the presence of transferrin in the serum. The use of serum-free medium overcomes this problem, but it has been found that RPMI-1640, the most commonly used culture medium for lymphocytes, contains a small but significant amount of endogenous iron (Brock, 1981) which is sufficient to saturate some of the transferrin added to the medium. This may therefore interfere with any attempt to study the role of apotransferrin. In order to determine whether transferrin is important in lymphocyte transformation as a membrane-triggering agent and not as an iron donor, it is necessary to determine whether apotransferrin promotes transformation. The problem of endogenous iron can, however, be overcome by adding a suitable iron chelator to the medium prior to the addition of apotransferrin. Desferrioxamine is an iron chelator with an iron-binding affinity constant greater than that of transferrin (Pollack et al., 1976). If DFO is used in the culture medium prior to the addition of apotransferrin

and the effects of transferrin on lymphocyte proliferation in the presence or absence of desferrioxamine are compared, the importance of transferrin as a membrane-triggering agent can therefore be demonstrated. However, this strategy would only work if the efficiency of apotransferrin as a trigger was similar to that of iron-containing transferrin. Some investigations along this line have already been reported, but they used either serum-containing medium (Novogrodsky et al., 1982) or a different culture technique (Brock, 1981), hence it was felt desirable to carry out further experiments using the controlled conditions described in Chapter 2 of this thesis.

2. The effect of cobalt- and manganese-containing transferrins on lymphocyte proliferation

The use of desferrioxamine to investigate the possibility that lymphocyte proliferation in vitro depends on a signal derived from the interaction of transferrin with its receptor, and that transferrin is not important as an iron donor, will only provide useful results if both apoTf and iron containing transferrins could cause a signal for proliferation. However, it could be that the induction of the triggering effect requires the conformation of transferrin induced when metal is bound. In order to test this possibility transferrin which had bound either cobalt or manganese was used instead of iron-containing transferrin. Cobalt and manganese are metals of the same transition series as iron and therefore have similar physico-chemical

properties. Since the ionic radii of cobalt and manganese ( $0.65 \text{ \AA}$  and  $0.62 \text{ \AA}$ , respectively), do not differ much from that of iron ( $0.67 \text{ \AA}$ ), they should be able to substitute for iron in the transferrin molecule without greatly altering its configuration. Complexes of transferrin with manganese or cobalt have been prepared previously (Aisen et al., 1969; Tan & Woodworth, 1969; Zweier et al., 1981). Since  $\text{Mn}^{3+}$  and  $\text{Co}^{3+}$  have been shown to occupy the same binding sites in the transferrin molecule as  $\text{Fe}^{3+}$  (Aisen et al., 1969; Tomimatsu et al., 1976) it is physiologically feasible to use them instead of iron. By using cobalt- or manganese-containing transferrins instead of iron transferrin it may therefore be possible to show whether transferrin is important in lymphocyte transformation because of its ability to donate iron or because of a membrane triggering effect.

### 3. The effect of transferrin with abnormal iron-binding properties on lymphocyte proliferation

A variant transferrin has been isolated by Evans et al. (1982) with an abnormal C-terminal iron-binding site. This transferrin also has a lower association constant with the transferrin receptor which is reflected in a reduced rate of iron-donation to transformed lymphocytes (Young et al., 1984a). However these workers did not investigate the degree of transformation induced by the abnormal transferrin. The effect of the variant transferrin on lymphocyte proliferation could therefore be used to determine whether transferrin

enhances transformation by causing a triggering effect on the lymphocyte membrane. If the variant transferrin induces the same proliferative response as the normal transferrin, the triggering effect is probably not important. However, if the variant transferrin causes low proliferation, this could be due to either low membrane triggering effect or due to low iron uptake

#### 4. The effect of a monoclonal antibody to the mouse transferrin receptor on lymphocyte proliferation

It is known that membrane receptors specific for transferrin are expressed on mitogen activated peripheral blood lymphocytes (Phillips, 1976; Galbraith et al., 1980b) and that membrane binding of transferrin results in the delivery of iron to the metabolically active lymphocytes (Hamilton, 1983). However, if the interaction of transferrin with its receptor also causes a triggering effect leading to cell proliferation, then a monoclonal antibody to the transferrin receptor may, if an alternative source of iron is also provided, be able to mimic the effects of transferrin and induce the same proliferative response. The effects on in vitro lymphocyte transformation of a monoclonal antibody to the transferrin receptor in the presence or absence of chelated iron have therefore been compared with the effect of iron-containing transferrin.

#### 4.2. MATERIALS

4.2.1. Variant and normal human transferrins. Variant and normal human transferrins isolated from the plasma of a donor who is heterozygous for the variant were kindly provided by Dr R.W. Evans, Guy's Hospital Medical School, London. Variant and normal transferrin preparations were rendered iron free by dialysis against 0.02 M citrate buffer at pH 5.1 for 24 h, and dialysed again against d-DW before being freeze-dried. Stock solutions (0.5 mg/ml) of variant and human transferrins were prepared in PBS (containing 1%  $\text{NaHCO}_3$ ). For different saturations of the transferrins with iron, the appropriate amount of iron as ferric nitrilotriacetate complex was added (see 2.2.2.2). Human control transferrin solution was prepared from Behringwerke transferrin as described elsewhere (see 2.2.2.6).

4.2.2. Monoclonal antibody to transferrin receptor. Monoclonal antibody to the mouse transferrin receptor (Ab  $\alpha$  TfR; R17208) was kindly provided by Dr I. Trowbridge, Salk Institute, San Diego.

#### 4.3. METHODS

##### 4.3.1. Preparation of cobalt and manganese complexes of transferrin

For the preparation of the cobalt transferrin complex ( $\text{CoTf}$ ) the method of Aisen et al. (1969) was used. The 1:1 complex of cobalt with citrate was obtained by mixing

equal volumes of 0.01 M citric acid and 0.01 M cobaltous nitrate solutions (Analar grade, BDH) and then slowly adjusting the pH of the solution to 5.0 by careful addition of 1M NaOH. A 3-fold excess of citrate-cobalt complex was added to a 5% (w/v) solution of human apotransferrin in 0.1 M KCl - 0.05 M Tris buffer, pH 7.3. The preparation was then made 0.04 M in  $\text{NaHCO}_3$  and allowed to stand for 30 h at 4°C in the dark. Increments of 2  $\mu\text{l}$  of 0.6% (w/v) of hydrogen peroxide were then added until no further increase in the absorbance at 405 nm was observed. Since the absorbance at 405 nm was not further increased after the addition of 6  $\mu\text{l}$  of peroxide solution to a solution containing 25 mg of transferrin in 0.9 ml medium, it was decided that the transferrin was fully saturated with iron. The spectrum of this solution exhibiting maximum at 405 nm, was similar to that obtained by Aisen et al. (1969). Unbound metal was removed by Sephadex G-25 gel filtration followed by passage of the preparation through a column of chelex-100 (Bio Rad. Laboratory, Richmond, Co., U.S.A.) equilibrated with KCl-Tris buffer. The chelex-100 did not remove any of the metal from the transferrin complex as indicated by the ratio of the absorbance of the transferrin solution at 280 and 405 nm which were the same before and after passing through the column. The final concentration of the transferrin in the solution was estimated by measuring the absorbance at 280 nm against KCl-Tris buffer and using the extinction coefficient for human iron-saturated transferrin at 280 nm;  $E_{1\text{cm}}^{1\%} = 14.1$  (Aisen et al., 1967),



since the extinction coefficient value for cobalt-saturated transferrin was unobtainable.

The manganese transferrin (MnTf) was prepared in the same way as the cobalt transferrin using  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  (ANALAR grade, BDH), and omitting the step of addition of peroxide. The concentration of transferrin in the solution and the percentage saturation with manganese were checked using the extinction coefficient values for iron-saturated transferrin at 280 nm ( $E_{1\text{cm}}^{1\%} = 14.1$ ; see above) and the extinction coefficient values for manganese-saturated transferrin at 429 nm ( $E_{1\text{cm}}^{1\%} = 1.09$ , Feeney & Komatsu, 1966). The percentage saturation of the transferrin with manganese was calculated using the formula:  $\frac{\text{OD}_{429}}{\text{OD}_{280}} \times \frac{14.1}{1.09} \times 100$

The spectrum of transferrin saturated with manganese prepared as described above exhibited a maximum at 429 nm and it was similar to the spectra obtained by other investigators (Feeney & Komatsu, 1966; Aisen et al., 1969).

#### 4.3.2. Lymphocyte transformation assay

Lymph node cell-suspensions were prepared and cells were cultured as described elsewhere (see 2.3.2). The proliferative responses were measured as in Section 2.3.2.

##### 4.3.2.1. Effect of desferrioxamine on the transformation of mouse lymphocytes in serum-free medium

Cells were cultured with ConA (0.5  $\mu\text{g}/\text{ml}$ ; see 2.4.2) in serum-free medium to which apotransferrin (50  $\mu\text{g}/\text{ml}$ ) and increasing concentrations of DFO (25 to 250  $\text{ng}/\text{ml}$ )

were added. The DFO was added to the culture medium 20 min prior to the addition of apotransferrin and cells to allow complete binding of endogenous iron. Controls without ConA or without DFO, and also a positive control with 25%-iron-saturated transferrin, were included. The reason for using 25%-iron-saturated transferrin as a positive control instead of 30%-iron-containing transferrin was that, at the time of this experiment, a new batch of culture medium was used (see 2.3.1.1) without knowing the levels of endogenous iron.

#### 4.3.2.2. Culture of cells with cobalt- or manganese-containing transferrin

Cells were cultured with ConA (1  $\mu\text{g/ml}$ ) in serum free medium to which transferrin saturated to varying degrees with iron, manganese or cobalt (see 4.3.1) was added.

#### 4.3.2.3. Culture of cells with variant and normal human transferrin

Lymphocytes were cultured with ConA (0.5  $\mu\text{g/ml}$ ) in serum-free medium containing either variant or normal human transferrin isolated from the same donor, or human control (Behringwerke) transferrin. Two different experiments were performed depending on the concentration of the transferrin used (i.e. either 50  $\mu\text{g/ml}$  or 5  $\mu\text{g/ml}$ ). Cultures were set up containing each transferrin as either apotransferrin or 30%-or 80%-iron-saturated transferrin. The cells were cultured in medium containing 80%-iron-saturated transferrin, because at this saturation both sites of transferrin will

contain iron and therefore it is ensured that the abnormal site will carry iron.

#### 4.3.2.4. Effect of anti-transferrin receptor monoclonal antibody on the transformation of mouse lymphocytes

Cells were cultured with ConA (0.5  $\mu\text{g/ml}$ ) in serum-free medium containing either 7.5 ng/ml or 15 ng/ml of iron as Fe(NTA) in the presence or absence of Ab  $\alpha$  TfR (50  $\mu\text{g/ml}$ ). The amount of iron added as Fe(NTA) (i.e. 7.5 and 15 ng/ml) was equivalent to that of 10%-or 20%-iron-saturated transferrin, respectively. The monoclonal antibody was added to the medium prior to the addition of Fe(NTA). Controls consisting of cells cultured alone or with Ab  $\alpha$  TfR only and also with apotransferrin and 10%-or 20%-iron-saturated transferrins were included. In these experiments, cell-cultures were set up in octuplicate with triplicate ConA-negative controls.

#### 4.3.3. Manganese and iron uptake from doubly labelled transferrin by proliferating lymphocytes

##### 4.3.3.1. Adjusting counting windows in the $\gamma$ -counter for dual counting of iron and manganese

Examination of the range of energy emitted by  $^{54}\text{Mn}$  was found, using a spectral plot, to overlap the range of emission by  $^{59}\text{Fe}$  (Fig. 9), so that in simultaneous counting of radiation emitted by the two metals, the counting of the energy emitted by one may also include energy emitted by the other. It is important, therefore, before measuring simultaneous uptake of manganese and iron to choose counting windows for each isotope which allow as much radiation as

Figure 9: Energy spectra of  $^{59}\text{Fe}$  and  $^{54}\text{Mn}$ .



The spectra of  $^{59}\text{Fe}$  (—) and  $^{54}\text{Mn}$  (---) were obtained by using 250nCi  $^{59}\text{Fe}$  and 250 nCi  $^{54}\text{Mn}$ .

possible emitted by each isotope to be counted but at the same time giving minimum spill over from one channel to the other. For this reason, before proceeding to the experiments concerning manganese and iron uptake by proliferating lymphocytes, the counting windows in the  $\gamma$ -counter (LKB Compugamma Model 1282 with 3" detector) were adjusted accordingly using a set of standards. The standards included: high and low activity (i.e. about 10,000 and 1,000 c.p.m., respectively) of  $^{54}\text{Mn}$  and  $^{59}\text{Fe}$ , and a mixture of high activity of  $^{59}\text{Fe}$  and low activity of  $^{54}\text{Mn}$ , and vice versa. Figure 9 shows the energy spectra for iron and manganese at window range of 200-240. For simultaneous counting of both metals, the windows were adjusted to 211-220 for manganese and to 222-235 for iron. The spillover which was 0.2% for  $^{54}\text{Mn}$  in the iron-channel and 12% for  $^{59}\text{Fe}$  in the manganese-channel, was corrected automatically by the machine.

#### 4.3.3.2. Estimation of manganese and iron taken up by proliferating lymphocytes

Mouse lymph node cells were cultured with ConA (0.5  $\mu\text{g}/\text{ml}$ , see 2.4.2) in serum-free medium containing transferrin doubly labelled with  $^{54}\text{Mn}$  (specific activity =  $94\mu\text{Ci}/\mu\text{g Mn}$ ) and  $^{59}\text{Fe}$  (specific activity 9-10 $\mu\text{Ci}/\mu\text{g Fe}$ ) (Amersham). Three different preparations of doubly labelled transferrin were obtained, each one having a total saturation with metal of 60%. However, the ratio of manganese to iron in each preparation was as follows: firstly, 3:1; secondly, 1:1 and thirdly, 1:3, taking into account also the iron content

of the medium (5 ng/ml). Preliminary work showed that 240 nCi of  $^{59}\text{Fe}$  and 800 nCi of  $^{54}\text{Mn}$  per  $3.6 \times 10^7$  viable cells gave adequate counts for measuring uptake of each metal by the cells after two days of culture. For the preparation of the double labelled transferrin, the radioactive metal was diluted, appropriately, first with the cold metal, and then mixed with the correct amount of transferrin solution (30  $\mu\text{g}$  of human transferrin/ml medium) for 10-15 min. before being added to the culture medium. Ferric nitrilotriacetate was used as a source of cold iron, and  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  for manganese, prepared in citrate buffer (Mn: citrate molar ratio 1:1, pH 5.0). The cells ( $2 \times 10^6$  viable/ml) were cultured at  $37^\circ\text{C}$  in an atmosphere of 90% air, 10%  $\text{CO}_2$  in 50 ml tissue culture flasks (see 2.3.1.2) in a total volume of culture medium of 18 ml. After 65 h of incubation the cultures, set up in duplicate, were spun (1200 r.p.m., 90 sec), the cells were washed three times in RPMI-1640 containing Hepes, and the  $^{59}\text{Fe}$  and  $^{54}\text{Mn}$  radioactivity associated with the supernatant, washes and the cells counted in the  $\gamma$ -counter using the windows described in Section 4.3.3.1. Finally, the percentage uptake of each metal by the cells was calculated. To determine whether different degrees of transformation took place at the three different proportions of metals, and because radioactive iron and manganese interfere with measurement of uptake of labelled thymidine, separate parallel cultures were set up in which the cells were cultured with transferrin containing cold iron and manganese at the three different

ratios described above, plus control containing 60%-iron-saturated transferrin. After 65 h of incubation, 1 ml aliquots were taken and slides of the cells were prepared for differential staining (Leishman). The rest (1 ml) was aliquoted into a microtitre plate (100  $\mu$ l/well), pulsed with  $^{14}\text{C}$ -thymidine for 4 h and the proliferative responses measured as described elsewhere (see 2.3.2).

#### 4.3.4. Iron uptake by proliferating lymphocytes in the presence or absence of monoclonal antibody to transferrin receptor

Mouse lymph node cells were cultured with ConA (0.5  $\mu\text{g}/\text{ml}$ , see 2.4.2) in serum-free medium containing 15 ng/ml iron as Fe(NTA) with or without monoclonal antibody to transferrin receptor (50  $\mu\text{g}/\text{ml}$ ). The amount of iron added to the medium was equivalent to that of 20%-iron-saturated transferrin when the transferrin was used at a concentration of 50  $\mu\text{g}/\text{ml}$ . Cells were also cultured in medium containing 25%-saturated transferrin (this amount of iron includes also the endogenous iron). The reason for using 25%-iron-saturated transferrin as a positive control instead of 30%, is explained in Section 4.3.2.1. Unlabelled Fe(NTA) was mixed with  $^{59}\text{Fe}$  (specific activity: 10  $\mu\text{Ci}/\mu\text{g}$  Fe) and this labelled Fe(NTA) was either used as such in the culture media, or added to transferrin or to the monoclonal antibody before these were added to the medium. The final activity was 240 nCi/ $2 \times 10^7$  viable cells, in a total volume of 10 ml. The cells were cultured in duplicate flasks as described in Section 4.3.3.2. After 58 h of incubation, the cultures were centrifuged

and the cells washed as described elsewhere (see 4.3.3.2). Finally, the  $^{59}\text{Fe}$ -activity of supernatant, washes and cells was measured in the  $\gamma$ -counter and the percentage iron uptake calculated.



#### 4.4. RESULTS

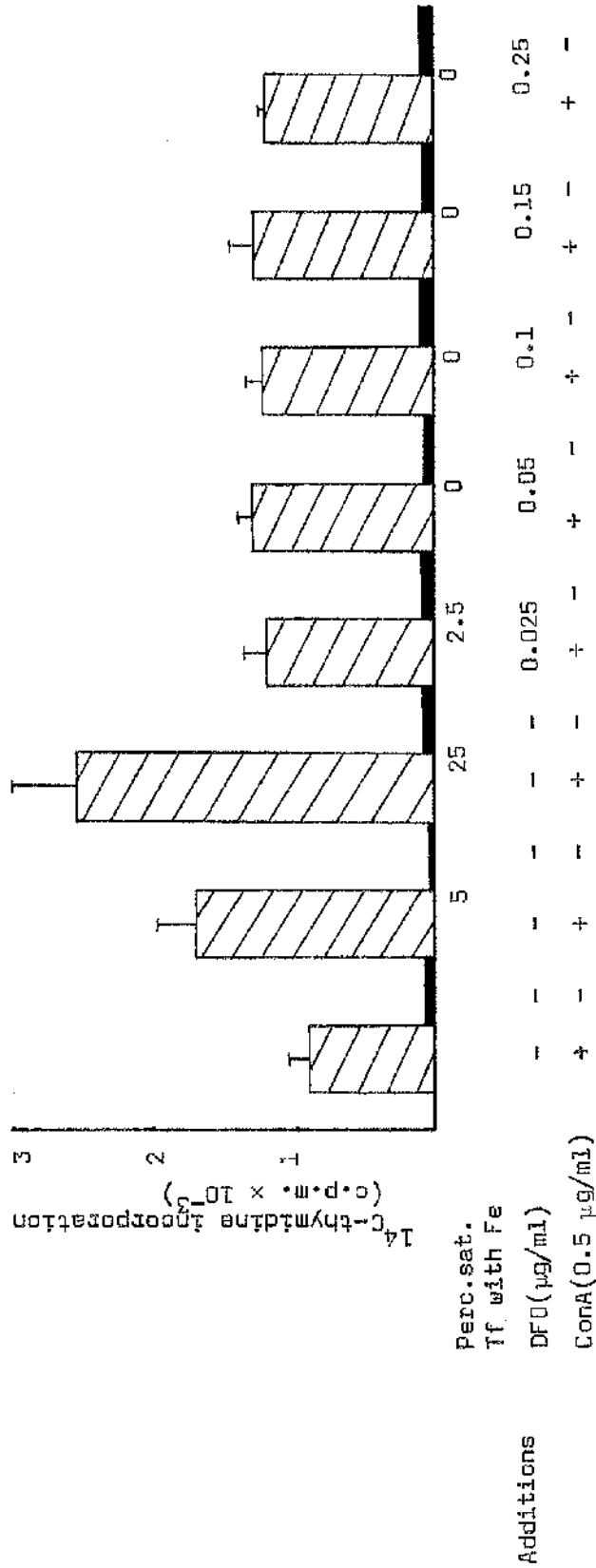
##### 4.4.1. The effect of desferrioxamine on the response to concanavalin A of mouse lymph node lymphocytes in serum-free medium

The degree of transformation of lymphocytes cultured in medium to which apotransferrin and DFO were added was significantly lower at all concentrations of DFO when compared with the responses of the cells cultured with transferrin but in the absence of DFO (Fig. 10, Table 36). The decreased proliferation may be due to DFO depriving transferrin and hence cells of the endogenous iron. However, the degree of proliferation at all concentrations of DFO was similar, and even the concentration which was enough to chelate only 50% of the endogenous iron (i.e. 0.025 µg/ml) caused the same reduction in transformation as higher concentrations of DFO sufficient to remove all the endogenous iron. It may be that at a concentration of DFO of 0.025 µg/ml, the amount of endogenous iron available to transferrin is so small that it does not cause any marked effect on the proliferative response.

##### 4.4.2. The effect of cobalt and manganese complexes of transferrin on the transformation of mouse lymph node lymphocytes in vitro

###### 4.4.2.1. The effect of cobalt-transferrin on the transformation of mouse lymphocytes in vitro

Figure 10: The effect of desferrioxamine on the transformation of mouse lymphocytes in serum-free medium.



Mean + SD. (+ConA, n = 7; -ConA, n = 4)

For statistical analysis, see Table 36.

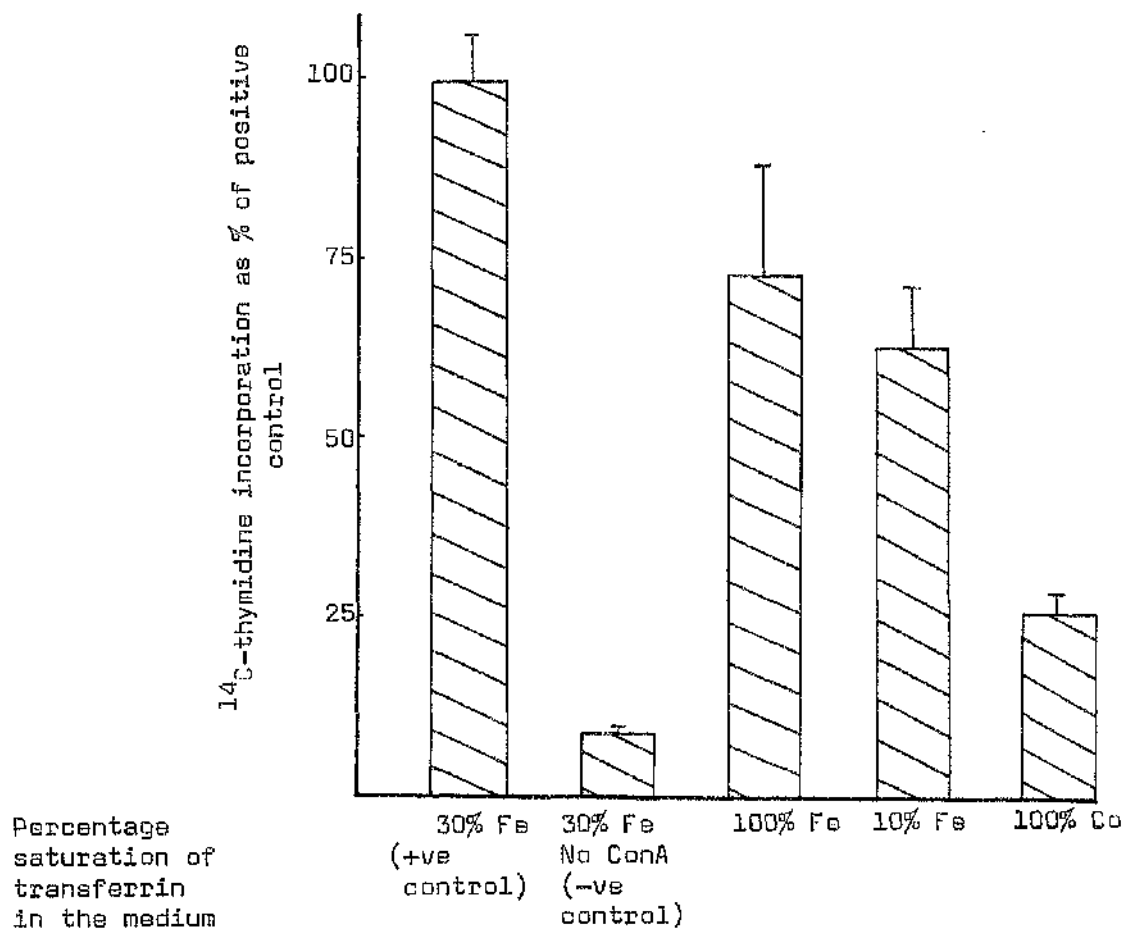
Table 36: Analysis by Scheffé's method of multiple comparisons of the effect of desferrioxamine on the transformation of mouse lymphocytes in serum-free medium (data from Fig. 10).

| Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|------------------|------------------------------------|------------------------|
| 1 and 2          | 631.90,434.00                      | S                      |
| 1 and 3          | -26.80,-567.22                     | S                      |
| 1. and 4         | -165.34,-680.66                    | S                      |
| 1. and 5         | -65.34,-580.66                     | S                      |
| 1 and 6          | -147.80,-688.23                    | S                      |
| 1 and 7          | -58.80,-599.25                     | S                      |
| 2. and 3         | 641.91,434.15                      | S                      |
| 2 and 4          | 502.65,311.44                      | S                      |
| 2 and 5          | 605.90,408.12                      | S                      |
| 2. and 6         | 510.90,313.11                      | S                      |
| 2 and 7          | 601.90,414.15                      | S                      |

\*1: cells cultured in absence of transferrin; 2: cells cultured in medium to which apotransferrin was added; 3,4,5,6,7: cells cultured in medium containing transferrin and DFO of concentrations of: 25, 50, 100, 150 and 250 ng/ml, respectively.

When mouse lymph node cells were cultured in medium containing cobalt-transferrin, the incorporation of labelled thymidine was significantly reduced in comparison with the proliferative response of the cells cultured in medium containing 100, 30, or 10%-iron-saturated transferrin (Fig. 11, Table 37). This result indicates that cobalt-containing transferrin does not promote lymphocyte proliferation as well as iron-containing transferrin, and that iron bound to transferrin is important for stimulated lymphocytes to proliferate in vitro. However, since iron displaces cobalt from the transferrin molecule (Zweier et al., 1981), the iron present in the medium (see 2.3.1.1) will displace some cobalt from the cobalt transferrin. The decreased proliferation of lymphocytes in medium containing 100%-cobalt-saturated transferrin may therefore be caused by free cobalt arising from the displacement of cobalt by endogenous iron. Also cobalt taken from transferrin by the cells may itself be toxic for the cells. To determine whether free cobalt was toxic, lymphocytes were cultured with ConA (0.5  $\mu$ g/ml) in medium containing 100%-iron-saturated transferrin plus cobalt (as cobaltous chloride solution made in d-DW) to give 5 ng of cobalt/ml of culture, which was equal to that displaced by iron. Cells were also cultured in medium containing 30%-and 100%-iron-saturated transferrin. Although free cobalt slightly decreased the lymphocyte proliferative response (Fig. 12), the effect was not significant (Table 38). This would indicate that

Figure 11: The effect of cobalt-transferrin and iron-transferrin on the transformation of mouse lymphocytes in serum-free medium.



Mean + SD. (+ConA, n = 14; -ConA, n = 8).

For statistical analysis, see Table 37.

Table 37: Analysis by Scheffé's method of multiple comparisons of the effect of cobalt-transferrin and iron-transferrin on the transformation of mouse lymphocytes in serum-free medium (data from Fig. 11).

| Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|------------------|------------------------------------|------------------------|
| 1 and 2          | -2301,-8719                        | S                      |
| 1 and 3          | 10935,4517                         | S                      |
| 1 and 4          | -12022,-18440                      | S                      |
| 2 and 3          | 5425,-993                          | NS                     |
| 2 and 4          | 2930,6512                          | S                      |
| 3 and 4          | -4296,-10714                       | S                      |

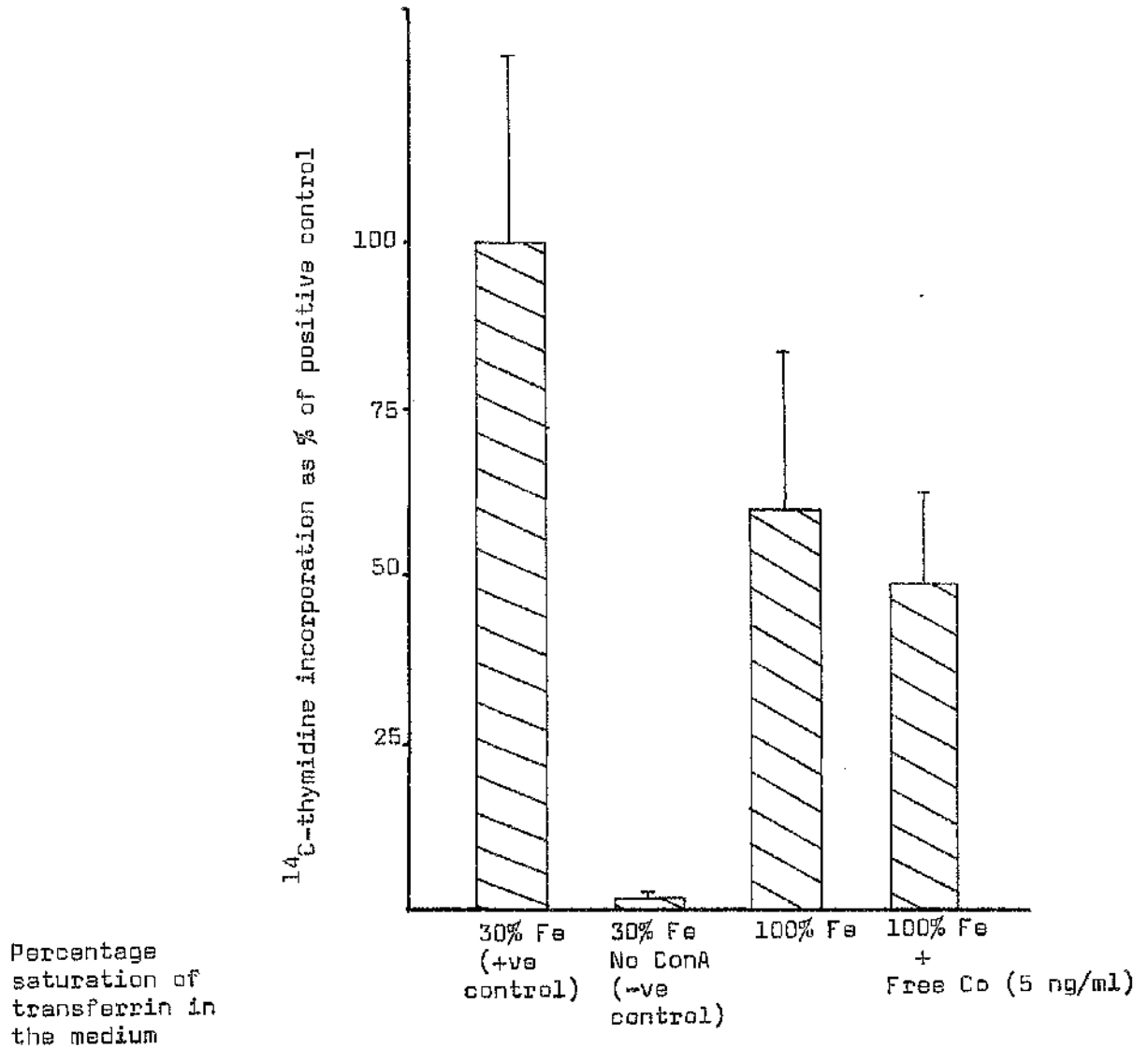
\*1: cells cultured in medium containing 30% FeTf; 2: cells cultured in medium containing 100% FeTf; 3: cells cultured in medium with 10% FeTf; 4: cells cultured in medium to which transferrin 100% saturated with cobalt was added.

reduced transformation of lymphocytes cultured in medium containing 100%-cobalt-saturated transferrin was probably not the result of a toxic effect of cobalt released into the culture medium. In order for the preparation of cobalt-transferrin, to ensure oxidation of  $\text{Co}^{2+}$  to  $\text{Co}^{3+}$  and binding of the metal to the transferrin molecule, hydrogen peroxide was used. Since hydrogen peroxide is a strong oxidising agent which, therefore, may affect the structure of transferrin, it was decided to use another metal which binds to transferrins with the same configuration and at the same binding sites but does not require peroxide for its preparation. Since manganese complex of transferrin has been prepared before without the use of peroxide (Ulmer & Vallee, 1963), it was therefore decided to use manganese.

#### 4.4.2.2. The effect of manganese-transferrin on the transformation of mouse lymphocytes in vitro

Cells were cultured with transferrin fully saturated with iron or manganese, and also in medium to which 100%-manganese-saturated transferrin plus free manganese, the amount was equivalent to that displaced by the endogenous iron (i.e. 12.5 ng  $\text{Mn}^{2+}$  as  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  solution, made in d-DW, per 1 ml of culture; this amount was added as the old batch of culture medium was used (see 2.3.1.1)). As with cobalt, the degree of transformation of mouse lymphocytes in medium containing transferrin fully saturated with manganese was significantly lower than that when 100%-iron-saturated transferrin was used (Fig. 13a, Table 39). This

Figure 12: The effect of free cobalt on the transformation of mouse lymphocytes cultured in serum-free medium containing transferrin-bound iron.



Mean + SD. (+ConA, n = 14; -ConA, n = 8).

For statistical analysis, see Table 38.



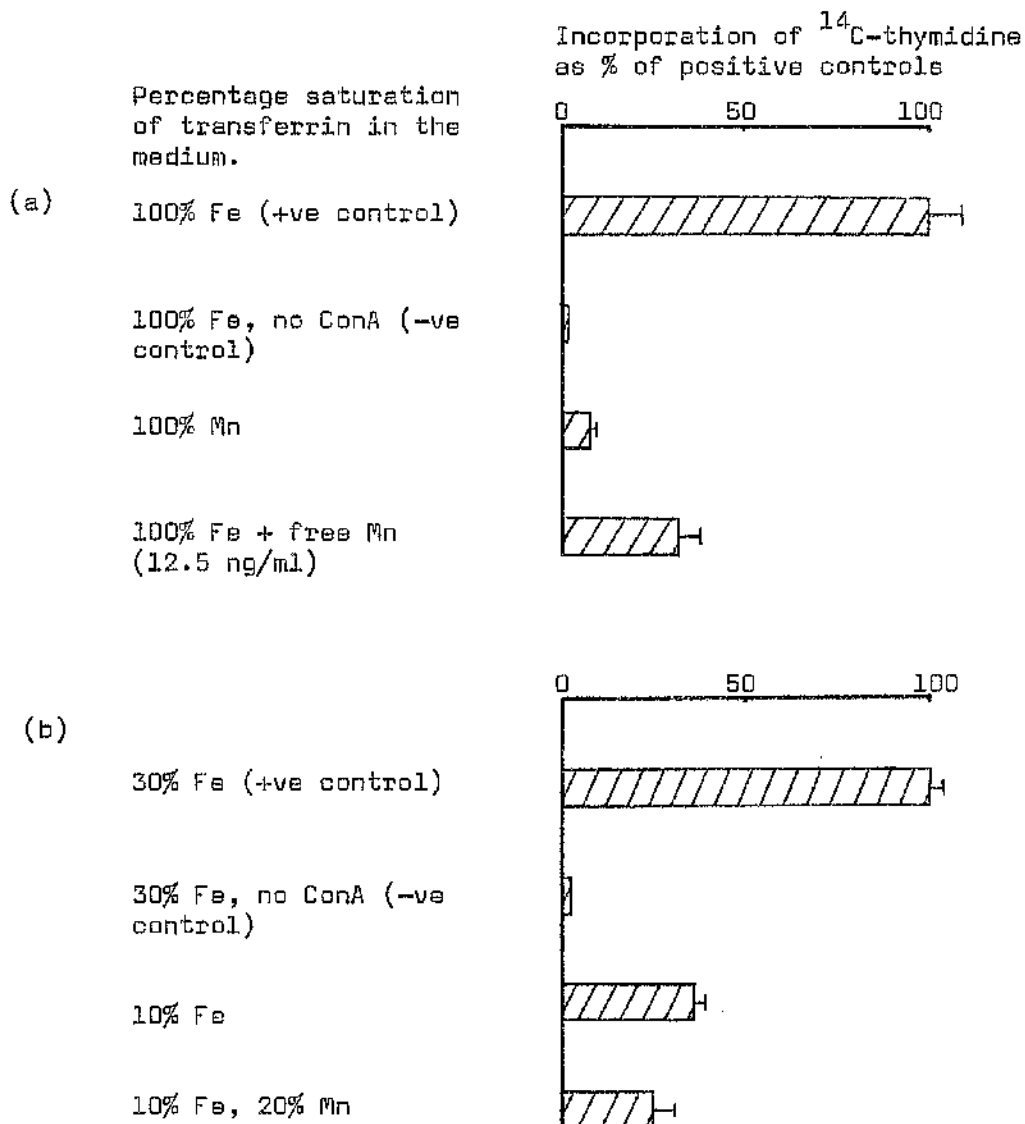
Table 38: Analysis by Scheffé's method of multiple comparisons of the effect of free cobalt on the transformation of mouse lymphocytes cultured in serum-free medium containing transferrin-bound iron (data from Fig. 12).

| Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|------------------|------------------------------------|------------------------|
| 1 and 2          | 6146.85,-2998.85                   | NS                     |
| 1 and 3          | -1297.15,-10442.95                 | S                      |
| 2 and 3          | -2871.15,-12016.85                 | S                      |

\*1: cells cultured in medium containing 100% FeTf; 2: cells cultured in medium containing 100% FeTf and free cobalt (5 ng/ml); 3: cells cultured in medium with 30% FeTf.

result indicates that manganese transferrin, like cobalt-transferrin, is not as good as iron-containing transferrin in promoting lymphocyte proliferation in vitro, and furthermore, do not support the idea that the proliferative response may depend on a signal derived from the interaction of the transferrin with its receptor, rather than simply the donation of iron. However, the degree of transformation in medium containing 100%-iron-saturated transferrin plus manganese equivalent to that displaced by the endogenous iron, was significantly lower (Fig. 13a, Table 39) than when 100%-iron-saturated transferrin was used alone, indicating that free manganese may have some adverse effects on cell proliferation. The low proliferative response, therefore, in the presence of 100%-manganese-saturated transferrin might have been partly due to the toxic effects of manganese released into the medium after being displaced by the endogenous iron, and this might have masked any triggering effect of manganese-transferrin. To avoid this problem, the effect of partially saturated transferrins was investigated. Cultures were set up in medium containing 30%-or 10%-iron-saturated transferrin, and also in medium to which apotransferrin and 100%-manganese-saturated transferrin were added in 4:1 ratio, respectively, so that the transferrin present in the medium had a saturation with metal of 20% manganese, 10% iron (derived from the endogenous iron of the culture medium), and 70% remained iron-free. This also compared the effect on cell proliferation of manganese-containing transferrin with a similar metal content

**Figure 13:** The effect of manganese-transferrin and iron-transferrin on the transformation of mouse lymphocytes in serum-free medium.



(a) Fully saturated transferrin (b) Partially saturated transferrin.

Mean + SD. (+ConA, n = 14; -ConA, n = 8).

For statistical analysis, see Tables 39 and 40.

Table 39: Analysis by Scheffé's method of multiple comparisons of the effect of free manganese on the transformation of mouse lymphocytes in serum-free medium (data from Fig. 13a).

| Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|------------------|------------------------------------|------------------------|
|                  |                                    |                        |
| 1 and 2          | 18760,15226                        | S                      |
| 1 and 3          | 14088,10692                        | S                      |
| 2 and 3          | -2836,-6370                        | S                      |

\*1: cells cultured in medium containing 100% FeTf; 2: cells cultured in medium containing transferrin 100% saturated with Mn; 3: cells cultured with 100% FeTf and free manganese (12.5 ng/ml).

Table 40: Analysis by Scheffé's method of multiple comparisons of the effect on the transformation of mouse lymphocytes of transferrin partially saturated with manganese or iron (data from Fig. 13b).

| Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|------------------|------------------------------------|------------------------|
| 1 and 2          | 15777,12413                        | S                      |
| 1 and 3          | 19102,14518                        | S                      |
| 2 and 3          | 5007,423                           | S                      |

\*1: cells cultured in medium containing 30% FeTf; 2: cells cultured with 10% FeTf; 3: cells cultured in medium containing 10% FeTf and 20% MnTf.

to 30%-iron-saturated transferrin. The degree of transformation in medium containing 10%-iron plus 20%-manganese-saturated transferrin was significantly lower than when cells were cultured in medium containing 30%-iron-saturated transferrin despite the fact that the content of metal-containing transferrin molecules was the same in both cases (Fig. 13b, Table 40). These results again suggest that the function of transferrin is mainly to supply iron to transforming lymphocytes and that a signalling effect caused by transferrin is not essential to promote transformation. However, the data could, alternatively, suggest that transferrin-bound manganese may be inhibitory per se. Manganese-transferrin could inhibit transformation by blocking transferrin receptors and hence uptake of iron. On the other hand, since transferrin with 10% iron and 20% manganese was less effective than transferrin containing 10% iron alone even though it contained a higher content of metal-bearing transferrin molecules, manganese-transferrin may also result in uptake of inhibitory amounts of manganese by the cells. In order to investigate whether the decreased proliferative response in the presence of manganese-containing transferrin might have been partly due to uptake of manganese by the cells, and also to determine whether the stimulated cells can distinguish between iron-containing and manganese-containing transferrin, studies of uptake of manganese and iron from transferrin by proliferating lymphocytes were performed.

4.4.2.3. Uptake of manganese and iron from transferrin by  
proliferating lymphocytes in vitro

At 1:3 ratio of iron to manganese, the amount of iron taken up by stimulated lymphocytes was as great as that of manganese, despite the greater quantity of manganese added. At higher iron to manganese ratios, the proportion of manganese taken up was further reduced. It seems therefore that at all ratios of iron to manganese, the cells preferentially take up iron (Table 41). The degree of transformation of stimulated lymphocytes and the proportion of blast-like cells was the same in the three media containing transferrin of different ratios of iron to manganese, indicating that enough iron was taken up at all three ratios of iron to manganese to induce the observed response and that the availability of iron was not a limiting factor (Table 42). However, the response of lymphocytes was much enhanced in medium containing 60%-iron-saturated transferrin (the saturation with iron was equivalent to the iron and manganese content of transferrins). Since cells take up manganese (Table 41), it seems that the decreased response in the presence of manganese may be due to toxic effects of manganese rather than blocking of the receptors.

4.4.3. The effect of variant and normal human transferrins  
on the proliferation of mouse lymphocytes cultured  
in serum-free medium

The degree of transformation of lymphocytes in medium to which variant transferrin was added either as apotransferrin

Table 41: Uptake of  $^{54}\text{Mn}$  and  $^{59}\text{Fe}$  by proliferating lymphocytes from doubly-labelled transferrin.

| Transferrin 60%<br>saturated with<br>metals in ratio | Fe taken up<br>(ng) |     | Mn taken up<br>(ng) |    | Ratio of uptake<br>Fe:Mn |       |
|--|---------------------|-----|---------------------|----|--------------------------|-------|
| Fe:Mn  | I*                  | II* | I                   | II | I                        | II    |
| 1:3  | 86                  | 80  | 83                  | 73 | 1.0:1                    | 1.1:1 |
| 1:1  | 127                 | 116 | 60                  | 53 | 2.1:1                    | 2.2:1 |
| 3:1  | 150                 | 141 | 41                  | 34 | 3.7:1                    | 4.1:1 |

\*Two different experiments were performed, I and II.



Table 42: The effect on the transformation of mouse lymphocytes of transferrin doubly-labelled with  $^{54}\text{Mn}$  and  $^{59}\text{Fe}$ .

| Transferrin saturation | $^{14}\text{C}$ -thymidine incorporation (c.p.m.)<br>$\text{I}^+$ | Percentage of blasts |
|------------------------|---|----------------------|
| 15% Fe + 45% Mn        | $789 \pm 197$   | 65                   |
| 30% Fe + 30% Mn        | $998 \pm 76$  | 63                   |
| 45% Fe + 15% Mn        | $954 \pm 98$  | 73                   |
| 60% Fe                 | $1851 \pm 100$  | nd                   |

$^+$ Mean  $\pm$  SD; n = 10. Figures obtained using the same cells as in Experiment I, see Table 41.

**Table 43(a):** The effect of a variant and normal human transferrins (50  $\mu\text{g/ml}$ ) on the transformation of mouse lymphocytes in serum-free medium.

| <sup>14</sup> C-thymidine incorporation (c.p.m.)   |                       |                       |                       |                       |                       |                       |
|--|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Iron-saturation<br>of transferrin<br>in the medium | 5% FeF                |                       | 30% FeF               |                       | 80% FeF               |                       |
|  | Variant <sup>a</sup>  | Normal <sup>a</sup>   | Variant <sup>b</sup>  | Control <sup>b</sup>  | Variant               | Control               |
| +ConA <sup>+</sup>                                 | 1988 <sup>±</sup> 339 | 2003 <sup>±</sup> 365 | 1443 <sup>±</sup> 113 | 3316 <sup>±</sup> 268 | 2618 <sup>±</sup> 246 | 3541 <sup>±</sup> 268 |
| -ConA <sup>+</sup>                                 | 62 <sup>±</sup> 5     | 54 <sup>±</sup> 7     | 51 <sup>±</sup> 6     | 72 <sup>±</sup> 7     | 40 <sup>±</sup> 5     | 50 <sup>±</sup> 6     |

for statistical analysis, see Table 44(a).

$$+_{\text{Mean}} +_{\text{SD}}; n = 7 \text{ for } +\text{ConA}, n = 4 \text{ for } -\text{ConA}.$$

<sup>a</sup>Variant and normal transferrins isolated from plasma of donor who is heterozygous for the variant

<sup>b</sup> Behringwerke human transferrin.

or 25%-iron-saturated transferrin was not significantly different from the response of cells cultured with normal human transferrin at the corresponding iron saturations (Tables 43a and 44a). These results indicate that at low iron saturations, variant transferrin is as good as normal transferrin in promoting transformation of lymphocytes in vitro and also that enough iron was taken up from the variant transferrin to induce the same response as with normal transferrin. However, at higher saturations with iron (i.e. 80%), the proliferative response induced by the variant transferrin was significantly lower than the response induced by normal transferrin (Tables 43a and 44a), indicating that, at high iron saturations, the variant transferrin is not as good as normal in promoting transformation.

However, this experiment was performed only once due to lack of availability of the variant transferrin. Furthermore, since variant transferrin at 50 µg/ml is well above the amount required to saturate all receptors, abnormal binding may become more apparent if the variant transferrin is used at lower concentrations. The same experiment was therefore repeated using 5 µg/ml transferrin instead of 50 µg/ml. At this low concentration, taking into account the amount of iron present in the culture medium (5 µg/ml, see 2.3.1.1), transferrins when added to the medium as apotransferrin or 75%-iron-saturated transferrin, become 50 and >100 per cent saturated, respectively. The variant transferrin at both saturations caused a slightly lower proliferative response when compared with normal or control

**Table 43(b):** The effect of a variant and normal transferrins (5  $\mu\text{g/ml}$ ) on the transformation of mouse lymphocytes in serum-free medium.

| <sup>14</sup> C-thymidine incorporation (c.p.m.) |                       |                       |                      |                       |                       |                       |
|--|-----------------------|-----------------------|----------------------|-----------------------|-----------------------|-----------------------|
| Iron saturation of transferrin in the medium     | 50% FeF               |                       |                      | > 100% FeF            |                       |                       |
|  | Variant               | Normal                | Control              | Variant               | Normal                | Control               |
| Cells cultured in medium containing:             | 1881 <sup>±</sup> 231 | 2140 <sup>±</sup> 179 | 2196 <sup>±</sup> 96 | 2260 <sup>±</sup> 645 | 2575 <sup>±</sup> 266 | 3050 <sup>±</sup> 954 |
|  | +ConA <sup>+</sup>    | 55 <sup>±</sup> 7     | 60 <sup>±</sup> 7    | 68 <sup>±</sup> 8     | 74 <sup>±</sup> 35    | 72 <sup>±</sup> 31    |
|  |                       |                       |                      |                       |                       | 60 <sup>±</sup> 5     |
|  |                       |                       |                      |                       |                       |                       |

For statistical analysis, see Table 44(b).

$$+ \text{Mean} \pm \text{SD}; n = 7 \text{ for } +\text{ConA}, n = 4 \text{ for } -\text{ConA}.$$

Table 44(a): Analysis by Scheffé's method of multiple comparisons of the effect of variant and normal transferrins (50  $\mu$ g/ml) on the transformation of mouse lymphocytes in serum-free medium.

| Percentage saturation of transferrin in the medium | Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|--|------------------|------------------------------------|------------------------|
| 5% FeTf  | 1 and 2          | 356.0,-386.0                       | NS                     |
|  | 1 and 3          | 916.0,174.0                        | S                      |
|  | 2 and 3          | 931.0,189.0                        | S                      |
| 30% FeTf   | 1 and 2          | 63.4,-48.3                         | NS                     |
|  | 1 and 3          | 14.8,-514.8                        | NS                     |
|  | 2 and 3          | 224.8,-304.8                       | NS                     |
| 80% FeTf   | 1 and 2          | -390.8,-1025.2                     | S                      |
|  | 1 and 3          | -605.8,-1240.2                     | S                      |
|  | 2 and 3          | 102.2,-532.2                       | NS                     |

\*1: cells cultured in medium containing variant transferrin;

2: cells cultured with normal transferrin isolated from the same donor as the variant transferrin;

3: cells cultured with control human transferrin (Behringwerke).

Table 44(b): Analysis by Scheffé's method of multiple comparisons of the effect of variant and normal transferrins (5  $\mu$ g/ml) on the transformation of mouse lymphocytes in serum-free medium.

| Percentage saturation of trans-ferrin in the medium | Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|---|------------------|------------------------------------|------------------------|
| 50% FeTf  | 1 and 2          | 0.3,-518.3                         | ns                     |
|   | 1 and 3          | 44.3,-474.3                        | ns                     |
|   | 2 and 3          | 205.1,-293.1                       | ns                     |
| >100% FeTf  | 1 and 2          | 268.8,-1018.8                      | ns                     |
|   | 1 and 3          | 56.0,-1636.0                       | ns                     |
|   | 2 and 3          | 141.0,-1103.0                      | ns                     |

- \*1: cells cultured in medium containing variant transferrin;  
 2: cells cultured with normal transferrin isolated from the same donor as the variant;  
 3: cells cultured with control human transferrin.

transferrins but the difference was not significant (Tables 43b and 44b). At low concentrations of transferrin, therefore, when less transferrin is available to interact with its receptor, the proliferative response induced by variant or normal transferrin was the same at either high (i.e. > 100%) or lower (i.e. 50%) saturations with iron, i.e. the same result was obtained as when the variant and normal transferrins were used at concentrations of 50 µg/ml and lower iron-saturations.

4.4.4. The effect of monoclonal antibody to mouse transferrin receptor on the transformation of mouse lymph node lymphocytes in vitro

The degree of transformation of lymphocytes in medium containing Ab α TfR was significantly lower than the response induced by apotransferrin (Tables 45 and 46). A lower proliferative response in comparison with that induced by 15% or 25%-iron-saturated transferrin was also observed with Ab α TfR plus Fe(NTA) of iron content equivalent to that of the corresponding iron-containing transferrin (Tables 45 and 46). It appears, therefore, that Ab α TfR cannot substitute for transferrin even in the presence of Fe(NTA). Furthermore, the proliferative response in the presence of Ab α TfR was significantly lower than when the cells were cultured without transferrin indicating that Ab α TfR may actually be toxic to the cells. When both Ab α TfR and Fe(NTA) were used, the degree of transformation was also lower than that when Fe(NTA) was used alone. The reduction in transformation

**Table 45:** The effect of monoclonal antibody to mouse transferrin receptor on the transformation of mouse lymphocytes in serum-free medium.

| Sample Number | 14C-thymidine incorporation (c.p.m.)<br>in culture medium containing: | +ConA <sup>+</sup> |            |         |          | -ConA <sup>+</sup> |    |
|---------------|---|--------------------|------------|---------|----------|--------------------|----|
|               |   | I*                 | II*        | I       | II       | I                  | II |
| 1             | Cells only  | 476 ± 50           | 569 ± 45   | 48 ± 2  | 70 ± 3   |                    |    |
| 2             | Cells + Fe(NTA)(7.5 ng Fe/ml) <sup>†</sup>                            | 467 ± 37           | 625 ± 27   | 35 ± 2  | 40 ± 6   |                    |    |
| 3             | Cells + Fe(NTA) (15 ng Fe/ml) <sup>‡</sup>                            | 452 ± 31           | 594 ± 65   | 30 ± 2  | 34 ± 2   |                    |    |
| 4             | Cells + Ab α TfR  | 179 ± 31           | 178 ± 17   | 30 ± 2  | 33 ± 2   |                    |    |
| 5             | Cells + Ab α TfR + Fe(NTA)(7.5 ng Fe/ml) <sup>‡</sup>                 | 102 ± 9            | 105 ± 17   | 22 ± 2  | 25 ± 4   |                    |    |
| 6             | Cells + Ab α TfR + Fe(NTA)(15 ng Fe/ml) <sup>‡</sup>                  | 71 ± 6             | 64 ± 9     | 22 ± 3  | 22 ± 1   |                    |    |
| 7             | Cells + ApoTf   | 1562 ± 110         | 987 ± 94   | 89 ± 10 | 124 ± 16 |                    |    |
| 8             | Cells + 15%-FeTf  | 1929 ± 150         | 1413 ± 135 | 97 ± 6  | 123 ± 3  |                    |    |
| 9             | Cells + 25%-FeTf  | 1988 ± 131         | 1489 ± 124 | 98 ± 19 | 100 ± 9  |                    |    |

For statistical analysis, see Table 46.

<sup>†</sup> Amount of iron equivalent to that added to transferrin in sample 8.

<sup>‡</sup> Amount of iron equivalent to that added to transferrin in sample 9.

\* Two experiments performed, I and II.

† Mean ± SD; n = 8 for +ConA, n = 3 for - ConA.

Ab α TfR at 50 µg/ml in all samples.



Table 46: Analysis by Scheffé's method of multiple comparisons of the effect of monoclonal antibody to transferrin receptor on the transformation of mouse lymphocytes in serum-free medium (data from Table 45).

| Groups compared* | Simultaneous 95% confidence limits | Significant difference |
|------------------|------------------------------------|------------------------|
| 1 and 2          | 1850.0, -1670.0                    | ns                     |
| 1 and 3          | 200.0, -152.0                      | ns                     |
| 1 and 4          | 373.0, 121.0                       | S                      |
| 2 and 3          | 191.0, -161.0                      | ns                     |
| 2 and 5          | 547.2, 182.8                       | S                      |
| 3 and 6          | 557.0, 205.0                       | S                      |
| 4 and 5          | 259.2, -105.2                      | ns                     |
| 4 and 6          | 2.8, -68.0                         | ns                     |
| 5 and 6          | 213.2, -151.2                      | ns                     |
| 4 and 7          | -120.7, -1559.0                    | S                      |
| 5 and 8          | -1644.8, -2009.2                   | S                      |
| 6 and 9          | -1734.8, -2099.2                   | S                      |
| 7 and 8          | -191.0, -543.0                     | S                      |
| 7 and 9          | -243.8, -608.2                     | S                      |
| 8 and 9          | 123.2, -241.2                      | ns                     |

\*1: cells only; 2: cells + Fe(NTA) (7.5 ng Fe/ml); 3: cells + Fe(NTA) (15 ng Fe/ml); 4: cells + Ab $\alpha$ TfR; 5: cells + Ab $\alpha$ TfR + Fe(NTA) (7.5 ng Fe/ml); 6: cells + Ab $\alpha$ TfR + Fe(NTA) (15 ng Fe/ml); 7: cells + ApoTf; 8: cells + 15% FeTf; 9: cells + 25% FeTf.

in the presence of Ab  $\alpha$ TfR and Fe(NTA) compared with the response in the presence of Ab  $\alpha$ TfR only, appeared to depend upon the level of Fe(NTA), but the counts were very low (Table 45). Iron uptake studies showed that in the presence of Ab  $\alpha$  TfR and Fe(NTA) a smaller amount of iron was taken up by the cells than in the presence of Fe(NTA) alone (Table 47). These results suggest that in the presence of Ab  $\alpha$ TfR the amount of iron taken up from Fe(NTA), although very small, may, nevertheless, interfere with rather than promote cell proliferation. Furthermore, the degree of transformation of lymphocytes in medium containing iron as Fe(NTA) equivalent to that present in 15 or 25%-iron-saturated transferrin, was the same as the response of the cells cultured without iron, although the proliferative response was much reduced compared with that when 15 or 25%-iron-saturated transferrin was used (Table 47). The percentage of iron taken up by proliferating cells from Fe(NTA) was similar to that taken from iron-containing transferrin (Table 47). These results confirmed the earlier observations of Brock & Rankin (1981) that, although, iron from Fe(NTA) is taken up by stimulated lymphocytes in vitro, it does not promote transformation.

Table 47: Iron uptake by mouse lymphocytes cultured with concanavalin A in serum-free medium containing either transferrin or ferric nitrilotriacetate or monoclonal antibody to mouse transferrin receptor.

| Culture medium containing             | Percentage of iron uptake by proliferating lymphocytes |            |
|---------------------------------------|--|------------|
|                                       | I*   | II*        |
| Fe(NTA) <sup>†</sup><br>(15 ng Fe/ml) | 25.5 ± 3.5 <sup>‡</sup>                                | 18.5 ± 1.5 |
| 25% FeTf                              | 25.5 ± 1.5   | 23.8 ± 0.2 |
| Fe(NTA)(15 ng Fe/ml)<br>+<br>Ab α Tfr | 6.0 ± 0.6  | 6.0 ± 0.3  |

<sup>†</sup>Amount of iron equivalent to that of 20% FeTf

<sup>‡</sup>Mean of two readings ± SEM

\*Two different experiments were performed, I and II.

#### 4.5. DISCUSSION

It is now well established that transferrin is important for lymphocytes to proliferate in vitro (Tormey & Mueller, 1972; Dillner-Centerlind et al., 1979; Larrick & Cresswell, 1979a; Galbraith et al., 1980a; Brock, 1981). This requirement of transferrin in cell proliferation is known to depend on its ability to donate iron to the cells (Lipsky & Lietman, 1980; Brock & Rankin, 1981; Bomford et al., 1983). However there are also literature data which suggest that transferrin may be required in cell proliferation because of a property unrelated to its iron-binding and donation (Brock, 1981; Tanno et al., 1982). It may be that when transferrin binds to its receptor on the cell membrane of stimulated lymphocytes, it causes a signalling effect which then leads to the events of proliferation. Membrane-triggering mechanisms exist in many systems. The interaction of IL-2 with IL-2 receptors on the membrane of activated T-lymphocytes initiates proliferation (Palacios, 1982a; Neckers & Cossman, 1983). The mitogenic or antigenic stimulation of lymphocytes also depends on a triggering effect caused as a result of the binding of the mitogen or antigen to their receptors on the cell membrane (Chess et al., 1976; Crumpton et al., 1976). It may be, therefore, that lymphocytes in order to proliferate also require a signal caused by transferrin interacting with the transferrin receptor on the membrane of stimulated lymphocytes.

The work reported in this chapter has attempted to define more closely the role of transferrin in the proliferation of lymphocytes in vitro with particular reference to its role as an iron donor or as a possible membrane triggering agent. This has been achieved by investigating the effects on cell proliferation of:

- 1) apotransferrin;
- 2) transferrin containing metals other than iron which bind to transferrin specifically and induce a similar conformation of the transferrin molecule to iron-transferrin;
- 3) a variant transferrin which has abnormal iron-binding properties and a lower affinity for the transferrin receptor than normal transferrin;
- 4) a monoclonal antibody to the mouse transferrin receptor in the presence or absence of inorganic iron.

If a membrane triggering mechanism caused by transferrin is important in the proliferation of lymphocytes, the apotransferrin itself may induce some degree of transformation, assuming that the cells can utilise their own intracellular iron. However, since endogenous iron in the culture medium will saturate some of the transferrin, DFO was used in order to render this iron non-available to transferrin. The degree of transformation of lymphocytes when apotransferrin and DFO were added to the medium was significantly higher than the response observed in the absence of apotransferrin and DFO (Fig. 10, Table 36). This suggests that lymphocyte transformation may depend on an interaction of transferrin with the cell-membrane which may then trigger events leading

to proliferation. However, since the increased transformation in the presence of apotransferrin was only slightly (though significantly) higher than that observed in the absence of transferrin and lower than that observed in the presence of 25%-iron-saturated transferrin, it may be that apotransferrin is not as good a triggering agent as iron-containing transferrin because it does not possess the same configuration as transferrin with iron bound. However, this difference could also be due to the role of iron-containing transferrin as an iron donor, so that the above result is not conclusive. To avoid this problem, the effect of cobalt- and manganese-containing transferrins were investigated, since these complexes should permit the effect of transferrin in the metal-containing conformation to be studied. Transferrin fully saturated with cobalt or manganese caused a significant reduction in the proliferative response in comparison with that induced by iron-saturated transferrin (Figs. 11 and 13a). However, culture medium to which transferrin 100% saturated with either cobalt or manganese is added will in fact contain transferrin which is 90% saturated with cobalt or manganese and 10% saturated with iron, due to endogenous iron displacing the cobalt or manganese. It will also therefore contain free cobalt or manganese (12.5 ng/ml, see 2.3.1.1). Since the response of lymphocytes in medium containing transferrin 100% saturated with cobalt or manganese was also significantly lower than that caused by 10%-iron-saturated transferrin, it may be that free cobalt or manganese are toxic to the

cells, and that this may mask a possible triggering effect of transferrin. To test this, a further experiment was performed in which either cobalt or manganese was added to the medium containing 100%-iron-saturated transferrin, in an amount equivalent to that being displaced by the endogenous iron. The free manganese, and to a lesser extent cobalt, caused a reduction in the transformation-promoting activity of 100%-iron-saturated transferrin, thus indicating that the free metals themselves caused some reduction in proliferation (Figs. 12 and 13b). To avoid the problem of toxicity caused by the free metal, partially saturated transferrins were used. Transferrin saturated 20% with manganese and 10% with iron still significantly reduced the proliferative response of lymphocytes in comparison with that induced by 30%-iron-saturated transferrin despite the presence of the same number of metal-containing transferrin molecules (Fig. 13b, Table 40). Furthermore, proliferation in medium containing 10% iron and 20% manganese was also significantly less than that observed when 10%-iron-saturated transferrin was used (Fig. 13b), despite the fact that both cultures contained the same number of iron-bearing transferrin molecules. This suggests that transferrin may not promote transformation by inducing a membrane signalling effect. However, the reduced response observed in the presence of manganese-transferrin may be due to manganese-transferrin binding to the transferrin receptors and thus preventing binding of iron-transferrin or alternatively, to a toxic effect of manganese acquired by the cells from transferrin.

Studies of uptake of manganese and iron by proliferating lymphocytes from transferrin doubly labelled with the two metals were therefore performed. These experiments showed that the cells can take up manganese from transferrin, suggesting that the low response of the cells in the presence of manganese-transferrin could be partly the result of intracellular toxic effects of manganese (Table 41). The proportion of iron taken up at the ratio of Fe:Mn of 1:3 was as high as that of manganese despite the fact that there was more manganese present, and at higher ratios of Fe:Mn, the iron taken up by the cells increased proportionally. It seems therefore that the cells take up iron preferentially. In addition, the percentage of blast cells, and the degree of transformation at all ratios of Fe:Mn was the same (Table 42), but reduced in comparison with that induced by transferrin 60%-saturated with iron. Taken together these observations suggest that the impaired cell proliferation in the presence of manganese-transferrin was not the result of inadequate iron uptake due to blocking binding of iron-containing transferrin, but more likely due to a toxic effect of manganese-transferrin. Additionally, since iron was taken up preferentially at all ratios of Fe:Mn it may be that manganese-transferrin has a lower binding affinity to the receptor than iron-containing transferrin. Although none of these experiments conclusively showed that transferrin does not act as a membrane triggering agent none gave any evidence that such an effect was occurring.



Further evidence for this conclusion came from studies using the variant transferrin. This transferrin binds iron abnormally at the C-terminal site, the complex being less stable than normal transferrin (Evans et al., 1982) and in addition, variant transferrin saturated with iron binds to the transferrin receptor with an affinity of one order of magnitude less than that of normal transferrin (Young et al., 1984a). If a membrane triggering effect is important, therefore, it would be expected that the reduced affinity of the variant transferrin for the receptor would result in reduced proliferation. At low iron-saturation, the variant transferrin caused a similar response to that of normal transferrin isolated from the same donor (Table 43a). This suggests that iron-availability was not a factor limiting cell proliferation in the presence of the iron and transferrin. However, at low saturation of transferrin with iron, it may be that no iron binds to the abnormal C-terminal site of the variant transferrin, and a difference between normal and variant might in consequence not be apparent. The experiment was therefore repeated using transferrin 80% saturated with iron, to ensure that some iron was bound at the C-terminal site of the variant transferrin, under these conditions. Since insufficient variant transferrin was available to repeat this experiment using 50 µg/ml of transferrin, a further experiment was carried out using the transferrin at 5 µg/ml. At this lower concentration, any effect of the lower affinity of the variant transferrin for the receptor should become more

apparent. At this condition, the proliferation of lymphocytes cultured in medium containing variant transferrin was significantly lower than that caused by normal transferrin (Tables 43a, 44a). However, at this concentration the variant transferrin also induced the same response as normal transferrin at high (> 100%) saturation with iron. The reduced response caused by variant transferrin at high iron-saturation and at 50 µg/ml in the previous experiment (see Table 43a) may have been an artifact. Nevertheless, since most results showed that the variant transferrin is as good as normal in promoting proliferation of stimulated lymphocytes it would appear that cell proliferation does not depend on a triggering effect caused by transferrin interacting with the transferrin receptor, and that the variant transferrin can, despite the lower affinity for the transferrin receptor, donate sufficient iron to lymphocytes in promoting optimal transformation.

An alternative approach to the problem of whether binding of a ligand to transferrin receptor provides a signal to initiate cell proliferation, is to use a ligand other than transferrin to bind to the receptor. The effect on lymphocyte transformation of a monoclonal antibody to the transferrin receptor was therefore investigated. When antibodies bind to specific cell receptors, they induce "patching" or "capping" leading to endocytosis. Patching of transferrin receptors on binding of transferrin to lymphocytes and endocytosis of the transferrin receptor complex has also been reported (Galbraith & Galbraith, 1980).

Thus, if binding of ligand, or patching of the transferrin results in the induction of a membrane triggering effect which leads to endocytosis and to events of proliferation then a monoclonal antibody to transferrin receptor should also mimic the effects of transferrin. However, Ab $\alpha$  TfR did not induce the same response as transferrin, at any iron saturation between 5 and 25%, even in the presence of a corresponding quantity of inorganic iron (Table 45). This result indicates that Ab $\alpha$  TfR cannot substitute for transferrin in enhancing proliferation, which in turn suggests that binding of transferrin or patching of transferrin receptors is not in itself a membrane triggering mechanism. However, the Ab  $\alpha$  TfR was not only unable to substitute for transferrin in promoting transformation but actually had inhibitory effects on proliferation compared with that observed when cells were cultured in the absence of transferrin. Possible mechanisms for this are discussed later.

In conclusion the results of experiments using cobalt or manganese transferrin, the variant transferrin and the monoclonal antibody to the transferrin receptor all suggest that the major role of transferrin is to donate iron to the cells. In no case was any evidence obtained to suggest that the interaction of transferrin causes a triggering effect leading to transformation which is independent of iron donation. However, in no case could it be unequivocally assumed that no triggering effect existed as other factors

could have influenced the results. Furthermore, in contrast to the other results, it was shown that the proliferation of lymphocytes was significantly higher in the presence of apotransferrin than in the absence of transferrin even when DFO was added to prevent any binding of endogenous iron to apotransferrin (Fig. 10). This could suggest that transferrin may enhance proliferation by triggering membrane events even when it cannot donate iron. However, some degree of proliferation was still observed even in the absence of (apo)transferrin. Sotys and Brody (1970) have shown that normal human peripheral lymphocytes synthesise transferrin. It may be, therefore, that transferrin is also required for some other function such as intracellular transport of iron, and that under adverse conditions when there is no extracellular transferrin-bound iron available, stimulated cells may be able to use endogenously synthesised transferrin to fulfil their needs, at least partially. Transferrin might be required intracellularly for the transport of iron from the labile pool to mitochondria, and it has been shown that isolated mitochondria take up labelled iron from transferrin (Konopka, 1978; Konopka & Turska, 1979). It may be possible, therefore, that the proliferative response observed when apotransferrin was used was not the result of a membrane triggering effect caused by the apotransferrin but that the (apo)transferrin is also required intracellularly. Although this is only a speculation, and the role of transferrin in the intracellular transport of iron remains to be resolved, it does

imply that the ability of lymphocytes to proliferate in the presence of apotransferrin may not necessarily be evidence for a membrane-triggering effect.

The effect of  $\text{Ab}\alpha\text{TfR}$  and  $\text{Fe(NTA)}$  in cell proliferation require some further discussion in addition to their inability to substitute for transferrin in promoting transformation.

It was found that in the absence of  $\text{Ab}\alpha\text{TfR}$ , the same amount of iron was taken up by the cells from  $\text{Fe(NTA)}$  as from transferrin (Table 47) although the degree of proliferation of stimulated cells in medium containing  $\text{Fe(NTA)}$  was significantly lower than that induced by the transferrin (Tables 45 and 46). Similar observations have been reported before (Brock & Rankin, 1981; Tanno *et al.*, 1982). Furthermore, no significant difference in the degree of proliferation was observed between cells cultured with or without  $\text{Fe(NTA)}$ . Taken together these observations indicate that  $\text{Fe(NTA)}$  cannot substitute for transferrin in promoting transformation. White and Jacobs (1978) showed increased uptake of iron from  $\text{Fe(NTA)}$  by Chang liver cells in comparison with that taken up from iron-containing transferrin. However, the iron taken up from  $\text{Fe(NTA)}$  was largely found to be membrane-associated and very little was incorporated into ferritin. In contrast, iron taken from transferrin was found to be mostly incorporated into ferritin. This suggests that iron acquired from  $\text{Fe(NTA)}$  probably does not enter the normal intracellular metabolic pathways. Since the other experiments reported in this section suggest that transferrin

does not promote proliferation by acting as a membrane triggering agent, it seems likely that Fe(NTA) fails to promote transformation not as a result of lack of a membrane triggering effect, but due to inaccessibility of the iron so acquired by cellular metabolism.

The fact that Ab $\alpha$ TfR actually inhibited transformation may be due to the fact that it is endocytosed via the same route as transferrin (Enns et al., 1983) and once inside the cell in the low pH of the endocytic vacuole, it may become dissociated from the transferrin receptor and then interfere with cell activity. Furthermore, the inhibitory effect of Ab  $\alpha$  TfR increased in the presence of inorganic iron (i.e. Fe(NTA)) and although the counts of the incorporated <sup>14</sup>C-thymidine were very low, the effect was reproducible and inhibition was proportional to the amount of inorganic iron added. Although the cells took up iron from Fe(NTA) in the presence of antibody, the quantity taken up was less than that taken in the absence of Ab $\alpha$ TfR (Table 47). The interaction of the Ab $\alpha$ TfR with the transferrin receptors may cause a reduction in membrane permeability in the stimulated lymphocytes. Since Ab $\alpha$ TfR plus Fe(NTA) is more inhibitory than Ab $\alpha$ TfR alone, and also since Ab $\alpha$ TfR is endocytosed via the same route as transferrin, it is suggested that inorganic iron in the presence of Ab $\alpha$ TfR is taken up via the same route as Ab $\alpha$ TfR rather than by diffusion across the membrane. Once inside the cell in the endocytic vacuole, the antibody, as explained above,

may become dissociated from the receptor and interfere with cell activity. The iron may or may not dissociate from NTA, but in either case the NTA may interfere with intracellular iron processing. To investigate such a possibility, it is necessary to study the fate of radio-labelled NTA in the cell in the presence and absence of Ab $\alpha$ TfR.

In conclusion, results presented in this chapter suggest that the importance of transferrin in the transformation of lymphocytes is not due to a membrane triggering effect which enhances proliferation as a result of interaction with receptors or inducing receptor patching. Transferrin is most likely required in cell proliferation solely for its iron-donating properties discussed in Chapters 2 and 3, possibly also for an undefined intracellular function.

#### 4.6. CONCLUSIONS

1. Apotransferrin has some transformation-promoting activity.
2. Transferrin containing either cobalt or manganese does not substitute for iron-transferrin as a transformation-promoting agent.
3. Variant transferrin with an abnormal C-terminal iron binding site is as good as normal transferrin in enhancing lymphocyte proliferation.
4. Monoclonal antibody to transferrin receptor cannot substitute for transferrin in promoting transformation even in the presence of inorganic iron.
5. The majority of these results indicate that transferrin enhances lymphocyte proliferation solely as a result of its ability to donate iron, and does not fulfil any additional function by triggering proliferation through a membrane signalling.



#### 4.7. SUMMARY

Although it is now well established that transferrin is required by proliferating lymphocytes, it is not clear whether this is due only to a requirement for the cells to acquire iron or whether the interaction of transferrin with a membrane receptor also acts as a signal for triggering transformation. In an attempt to answer this question, desferrioxamine, an iron chelator, was used to remove endogenous iron present in the culture medium and allow the effect of apotransferrin on lymphocyte transformation to be investigated. It was found that when enough desferrioxamine to chelate the endogenous iron was added to the medium prior to addition of transferrin the degree of transformation was significantly reduced when compared with the response of cells cultured in medium containing iron-transferrin. However the proliferative response in medium containing apotransferrin (i.e. in the presence of desferrioxamine) was significantly higher than when cells were cultured in the absence of transferrin indicating that transferrin without iron does have some transformation-promoting activity. In order to investigate further the relative importance of iron and transferrin in lymphocyte transformation the effects of transferrin containing iron ( $\text{Fe}^{3+}$ ) or other metals ( $\text{Co}^{3+}$ ,  $\text{Mn}^{3+}$ ) which form complexes with a similar configuration were compared. Transferrin 100% saturated with manganese or cobalt was markedly less effective than transferrin 100% saturated with iron, but this was probably due to

toxicity of free metal displaced from transferrin by iron present in the culture medium. However, transferrin saturated 20% with manganese and 10% with iron was also less effective than transferrin saturated to either 10% or 30% with iron alone. Studies using  $^{59}\text{Fe}$  and  $^{54}\text{Mn}$  bound to transferrin at different ratios showed that both manganese and iron were taken up by the cells, although iron was taken up to a greater extent, indicating that toxicity of manganese was more likely to be responsible for the low transformation-promoting ability of manganese transferrin than a blocking action of manganese-transferrin on iron uptake was more likely to be responsible. Nevertheless, these results do suggest that transformation-promoting activity of transferrin is dependent primarily upon its role as a source of iron rather than as a membrane triggering agent. Further evidence for this conclusion was provided by experiments showing that a variant human transferrin which binds to lymphocytes with lower affinity than normal transferrin nevertheless promoted optimum transformation. Furthermore, a monoclonal antibody to the mouse transferrin receptor could not substitute for transferrin as a receptor-triggering agent even when inorganic iron was provided in the medium.

#### GENERAL SUMMARY AND CONCLUSIONS

Transferrin-bound iron is important for lymphocyte proliferation. Evidence for this comes from the observations of the present investigation showing that the proliferation of mitogen-stimulated mouse lymph node lymphocytes depends on the availability of transferrin-bound iron both in serum-free medium and in medium containing serum from iron-sufficient or iron-deficient mice. The reduced proliferative response of lymphocytes cultured in medium containing serum from iron-deficient mice was the result of low levels of transferrin-bound iron since iron-repletion in vitro & in vivo corrected the defect. Furthermore, lymphocytes isolated from normal or iron-deficient and iron-sufficient mice proliferated at the same degree irrespective of the iron availability in the culture medium, indicating that iron deficiency did not cause any intrinsic defects in the ability of the lymphocytes to proliferate. The proportion of T-cells and T-cell subsets was also not altered in iron deficiency. Taken together these observations suggest that the reduced lymphocyte proliferation and perhaps therefore, the impaired DTH responses associated with iron deficiency are the result of decreased levels of circulating transferrin-bound iron rather than of intrinsic abnormalities in the lymphocytes or altered lymphocyte-subpopulations. In addition, the importance of transferrin in lymphocyte proliferation was shown to depend on its iron-donating

properties, rather than on a triggering effect caused by transferrin interacting with the transferrin receptor. This was concluded from experiments showing that transferrin containing cobalt or manganese which has a similar configuration to iron-transferrin did not enhance proliferation when compared with the response induced by iron-containing transferrin. Furthermore, a variant transferrin which has a lower receptor-binding affinity constant than normal transferrin induced the same response as normal transferrin, and a monoclonal antibody to transferrin receptor could not substitute for transferrin as a transformation-promoting agent even when inorganic iron was added. Thus, it can be concluded that the expression of transferrin receptors in proliferating lymphocytes is probably entirely related to the iron-requirements in the cells.

In conclusion, iron bound to transferrin is one of the controlling factors for the in vitro proliferation of lymphocytes. This is probably because iron is important for the activity of the enzyme ribonucleotide reductase which is required for the synthesis of DNA (Hoffbrand et al., 1976). How this compares with the in vivo situation is difficult to predict. Under normal conditions, there is a constant supply of circulating transferrin-bound iron, but in iron deficiency, the saturation of transferrin with iron is very much reduced so that in a localised area, such as a lymph node, consumption of iron bound to transferrin may not be replenished fast enough resulting in impaired immune response.

APPENDIX

STATISTICAL ANALYSIS

## STATISTICAL ANALYSIS

In biological sciences unlike the more exact sciences of physics and chemistry, inherent variation is very high. Since this variation can make numerical arguments more difficult and unreliable, it is necessary to describe the basic variability by using appropriate statistical methods so that valid and objective conclusions are reached.

The data presented in this thesis were analysed by methods appropriate to the experimental design and the problem to be resolved. The following methods have been used:

### 1. Student's t-test

This is a statistical method which is used to determine whether measurements on two samples taken from two populations are compatible with the null hypothesis that the two populations have the same mean, and it is achieved by comparing the difference between the two sample means with the estimated standard error of the difference. The test statistic  $t$  may be regarded as the difference between the two sample means divided by the standard error of the difference:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sigma^2 \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \sim t_{(1-\alpha)}(n_1 + n_2 - 2)$$

where  $\sigma^2 = \frac{\sum (X - \bar{X}_1)^2 + \sum (X - \bar{X}_2)^2}{n_1 + n_2 - 2}$  and  $n_1, n_2$  are the number of observations in samples  $S_1$  and  $S_2$  respectively.

The test is based on the following assumptions:

- i) the observations in each sample are independent
- ii) the samples show normal distribution
- iii) the variability within each sample is the same (i.e. have the same variances).

Suppose it is desired to know whether two populations (from which two samples are drawn) are different. Using t-test, the null hypothesis ( $H_0$ ) can be considered, and assuming this null hypothesis, the probability of obtaining a value of  $t$  equal or greater than the value actually observed can be calculated. If this probability is very small, (e.g.  $p < 0.05$ ) we can reject the null hypothesis with reasonable safety. The probability ( $p$ ) of obtaining or exceeding a given value of  $t$  with a particular sample-size can be most conveniently obtained from published tables. Alternatively, an acceptable level of uncertainty (a confidence criterion) such as  $p < 0.05$  may be chosen in advance, and the critical value of  $t$  that would be required to satisfy this criterion may be obtained from tables. If:  $t_{\text{observed}} > t_{\text{tabulated}}$ , then the null hypothesis is rejected in favour of the alternative hypothesis that the means are different at (say) 5% significant level (i.e.  $p < 0.05$ ). However, if  $t_{\text{observed}} < t_{\text{tabulated}}$ , then the null hypothesis is tenable and the means of the two populations from which the two samples are drawn may well be equal. This implies that there may be no difference between means of the two populations. It is important to note that a lower significance level can be used such as 0.01 (i.e. 1%) or 0.001 (i.e. 0.1%), instead of 5% level, especially if the alternative hypothesis is particularly unlikely a priori.

The t-distribution can also be used to attach "confidence limits" to estimates, so that at the accepted significance level (say 5%) it can be estimated that for  $\mu_1 - \mu_2$  (where  $\mu_1, \mu_2$  are the means of the two populations from which the samples are drawn):

$$\bar{x}_1 - \bar{x}_2 - t_a(n_1 + n_2 - 2) \sigma^2 \sqrt{\frac{1}{n_1} + \frac{1}{n_2}} < \mu_1 - \mu_2 < \bar{x}_1 - \bar{x}_2 + t_a(n_1 + n_2 - 2) \sigma^2 \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

where  $\bar{x}_1 - \bar{x}_2 \pm t_a(n_1 + n_2 - 2) \sigma^2 \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$  are the confidence limits

and  $t_a(n_1 + n_2 - 2) \sigma^2 \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$  the confidence interval.

Student's t-test is used to compare the means of two samples. If more than two samples are available it is unreliable to use the t-test because when the number of t-tests increases, the chance of an accidentally low p value increases proportionally.

## 2. Scheffé's method of multiple comparisons

This is a statistical method which is used to compare simultaneously the means of more than two samples taken from different populations. The method, which is based on the same assumptions as the t-test, states that a set of simultaneous 95% confidence intervals for the  $1/2 K(K-1)$  pairs ( $K$  = number of samples to be compared) of means

$\mu_i - \mu_j$  are given by:

$$\bar{x}_i - \bar{x}_j \pm \sqrt{(K-1) F_{0.05/K-1, N-K}} \times \sqrt{\sigma_{\text{pool}}^2 \left( \frac{1}{n_i} + \frac{1}{n_j} \right)}$$

where  $\bar{x}_i - \bar{x}_j$  is the difference of the estimated means of the samples  $S_i, S_j$ , respectively, and



$$1) \quad \sigma^2_{\text{pool}} = \frac{\sum_{i=1}^K (n_i - 1) \sigma_i^2}{N - K}$$

where:

$n_i$  = number of observations within  $S_i$

$\sigma_i^2$  = variance within  $S_i$

$N$  = total number of observations

2)  $F_{0.05/K-1, N-K}$  is the 5% points of variance-ratio (F) distribution at  $(N-K)$  degrees of freedom ( $f_1$ ) and  $(K-1)$  residual ( $f_2$ ).

So, for the actual difference of means  $\mu_i - \mu_j$  :

$$(i) \quad \bar{x}_i - \bar{x}_j - \sqrt{(K-1)F_{0.05/K-1, N-K}} \times \sqrt{\sigma^2_{\text{pool}} \left( \frac{1}{n_i} + \frac{1}{n_j} \right)} < \mu_i - \mu_j \text{ and}$$

$$(ii) \quad \mu_i - \mu_j < \bar{x}_i - \bar{x}_j + \sqrt{(K-1)F_{0.05/K-1, N-K}} \times \sqrt{\sigma^2_{\text{pool}} \left( \frac{1}{n_i} + \frac{1}{n_j} \right)}$$

There are three possibilities:

1) If the first part of the inequation (i) above and the second part of (ii) are both positive numbers, then:

$\mu_i - \mu_j > 0$  or  $\mu_i > \mu_j$  and therefore the null hypothesis that the two means are the same, is rejected in favour of the alternative hypothesis that there is a difference between the means of the two parent populations.

2) Similarly, if the first part of the inequation (i) and the second part of (ii) are both negative numbers, then:

$$\mu_i - \mu_j < 0 \text{ and therefore } \mu_j > \mu_i$$

In this case, as in the previous one, the null hypothesis is rejected and it is concluded that there is evidence that there is a significant difference between the means of the corresponding samples.

3) Either the first part of (i) is a positive number and the second part of (ii) is a negative number, or vice versa. In this case:

$$\begin{array}{ccc} \text{negative} & & \\ \text{number} & < \mu_i - \mu_j < & \text{positive number} \end{array}$$

This indicates that the difference  $\mu_i - \mu_j$  is a small number which may well be zero i.e.  $\mu_i - \mu_j = 0$  or  $\mu_i = \mu_j$

In this case the null hypothesis is accepted and it is concluded that there is no evidence that the means of the samples are different.

Scheffé's method of multiple comparisons has the property that, if the null hypothesis is true (i.e. all  $\mu_i$  are equal) in only 5% of experiments will it be possible to find a pair of means which satisfy the confidence criterion by accident (e.g. random sampling error). Thus the 95% confidence level applies to the experiment taken as a whole, and not to each pair of means taken separately.

### 3. Analysis of variance

This is a statistical technique in which the total variation of the numerical results about their mean is subdivided into components attributable to variations between the factor(s) (i.e. the variable(s) which is believed to affect the outcome of the experiment) and the residual variation attributable to variations between individual animals, errors of measurements etc.

The two-way analysis of variance (two-way ANOVA) is used to investigate the effect of two different factors; A and B, each one having a number of different levels, say h and g, respectively.

The errors are assumed to be normally and independently distributed, random variables with mean zero and variance  $\sigma^2$ . It can be shown that the total variation can be

subdivided into three parts; one for each of the two factors and a third being the residual variation. Thus:

Total sum of squares = factor A sum of squares + factor B sum of squares + residual sum of squares.

OR:  $SST = SSA + SSB + SSE$

where  $SST = \sum_{i=1}^h \sum_{j=1}^g Y_{ij}^2 - C.F.$  C.F. (i.e. correction factor) =  $Y^2/N$

$SSA = \frac{1}{g} \sum_{i=1}^h Y_i^2 - C.F.$   $Y = \text{sum of all responses}$   
 $Y_i = \sum_{j=1}^g Y_{ij}$

$SSB = \frac{1}{h} \sum_{j=1}^g Y_j^2 - C.F.$   $Y_j = \sum_{i=1}^h Y_{ij}$

$SSE = SST - SSA - SSB$

There are three variances:

$\sigma_2^2 = SSA/h - 1$  ,  $\sigma_3^2 = SSB/g - 1$  ,  $\sigma_1^2 = SSE / (h-1)(g-1)$

Considering the null hypothesis of no difference between the levels for factor A, there are two estimates of variances:  $\sigma_1^2$  and  $\sigma_2^2$  . If the F estimated value for factor A ( $F = \sigma_1^2 / \sigma_2^2$  ) is greater than the critical statistic value F (obtained from tables for F-distribution) on  $[(h-1), (h-1)(g-1)]$  degrees of freedom and at a certain level of confidence (i.e. 0.95 or 0.99 or 0.999), then there is evidence to reject the null hypothesis of no difference between any levels of factor A and to accept the alternative hypothesis of some difference. Similarly, if  $\sigma_3^2 / \sigma_1^2$  is

greater than F critical value at  $[(h-1), (h-1)(g-1)]$  degrees of freedom, then the null hypothesis is rejected again in favour of the alternative hypothesis.

When several replicate samples have been tested with the same levels of factors A and B, it is also possible to separate the residual sum of squares further into two components: an error sum of squares arising from differences among replicates treated identically, and an interaction sum of squares arising from the interaction between factors A and B. If a significant interaction is observed this implies that the effects of A and B are not simply additive: the effect of A is increased at particular levels of B or vice versa. There may, for example, be synergy between A and B, or conversely, particular levels of one factor may inhibit the effects of the other factor. The analysis may be extended, in principle, to any number of factors, and the mathematics then become exceedingly complex. For these analyses the computer programmes MINITAB and RUMMAGE were employed, using a Northstar Advantage computer.

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